The Development of a Human Cell Culture Assay for Skin Tumour Promoters

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by

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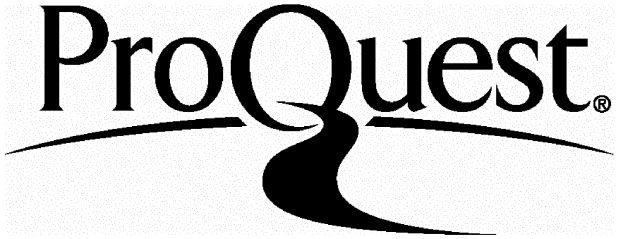

ABSTRACT

A valid model of initiated epidermis requires that normal keratinocytes should suppress the growth of initiated keratinocytes and that this suppression should be overcome on exposure to tumour promoters. Subgenomic SV40 transformed human keratinocytes (HK-4), which display the initiated phenotype, were cultured within a confluent monolayer of normal human keratinocytes and exposed to various tumour promoters and anti-promoters.

This system appeared to be successful in identifying both phorbol ester and non-phorbol ester type tumour promoters in a dose-dependent manner as determined by the size of the colonies of the HK-4 cells. The ranking of three phorbol ester-type tumour promoters matched the order of potency of these compounds as tumour promoters in mouse skin. In this model tumour promoters appear to induce the clonal expansion of HK-4 cells indirectly by selectively stimulating the differentiation of the normal cells.

The anti-promoters retinoic acid and fluocinolone acetonide were unsuccessful in inhibiting TPA induced colonies in this human model. A major reason for the absence of anti-promoter activity in the co-culture has been attributed to the abnormal conditions of the cells (i.e grown on plastic and submerged in medium). The presence of a dermis and an air-liquid interface is important in cellular functions such as differentiation and proliferation. Cells grown as organotypic cultures better represent the *in vivo* situation compared with those in submerged cultures. Therefore the co-culture of HK-4 and normal human keratinocytes was incorporated within a skin equivalent at the air-liquid interface to produce a three dimensional model of initiated human epidermis. Mimicking the situation *in vivo* may give rise to a more accurate interpretation of tumour promotion in human skin. In addition, this system allows test samples to be added either topically (placed on the surface of the skin equivalent) or systemically (placed in the medium below the dermis).

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Abbreviations

CRABP	Cellular retinoic acid binding protein
DAB	Diamino benzidine
DE	Dermal equivalent
DFMO	Difluoromethyl Ornithine
DMBA	Dimethyl benzanthracene
DNA	Deoxyribonucleic acid
EBS	Epidermolysis bullosa simplex
EGF	Epidermal growth factor
FA	Fluocinolone acetonide
GJIC	Gap junctional intercellular communication
GST	Glutathione-s-transferase
HF	Hair follicles
HFE	Hair follicle explants
K	Keratin
NGS	Normal goat serum
NHK	Normal human keratinocytes
ODC	Ornithine decarboxylase
ORS	Outer root sheath
PBS	Phosphate buffered saline
PKC	Protein kinase C
RA	Retinoic acid
RNA	Ribonucleic acid
SAP A	Sapintoxin A
SB	Skin biopsy
SE	Skin equivalent
TCDD	2,7,3,8-Tetrachloro-p-dibenzo dioxin
TGF	Transforming growth factor
TPA	12-O-Tetradecanoyl phorbol-13- acetate also know as Phorbol myristate acetate.
TPCK	Tosyl phenylalanine chloromethyl ketone

CHAPTER ONE:

INTRODUCTION

1.1 INTRODUCTION

1.1.1 Skin cancer statistics

Epithelial skin cancer is the most prevalent malignancy in humans with almost one million new cases estimated to occur each year in the United States (Darwiche 1995). Skin cancers account for 10% of all human neoplasms in Britain (HMSO 1985), with 40,000 new cases being reported each year in England and Wales alone (Cancer Research Campaign 1995).

It has been estimated that around 70% of cancers can be attributed to environmental factors such as diet and cigarette smoking (Williams 1988); exposure to solar UV radiation is the main environmental factor linked to skin cancer. There is however increasing evidence to suggest that environmental and occupational exposure to chemicals especially tars and oils can contribute to the development of tumours of the skin (Agarawal & Mukhtar 1992; Doll 1967).

1.1.2 Problems in identifying carcinogens

Currently, the only acceptable means for identifying chemical carcinogens involves long term and expensive rodent assays which can take up to 24 months to complete at an estimated cost of US$1.5 million per chemical. With the ever increasing number of chemicals being introduced into the environment by the chemical, pharmaceutical and agricultural industries, such long term assays have become inadequate. Apart from being time consuming it has become financially impossible to test each new chemical synthesised for commercial use without creating a huge backlog of untested compounds. Consequently, there has been a demand for short term, and preferably less expensive

assays, which could help to reduce the number of chemicals having to be tested in *in vivo* rodent assays.

1.1.3 Short term assays

A number of short term *in vitro* assays have been developed to identify carcinogens. Chemical carcinogens may be divided into those that initiate tumours and those that promote tumour formation (Berenblume 1941; Agarawal & Mukhtar 1992; Slaga 1983; Yuspa 1994). These stages are often equated with genotoxic and non-genotoxic mechanisms, respectively. Short term systems developed to identify genotoxic chemicals have proved to be successful as pre-screening assays e.g. the Ames *Salmonella Typhimurium* mutation assay, which is used widely to measure chemically induced mutations as a short term test for initiating carcinogens. However, there are very few short term *in vitro* assays to identify tumour promoters and tumour promotion has remained poorly understood in comparison. Nevertheless the importance of tumour promotion has reached the attention of regulators. For example, the Nordic Council of Ministers recently recommended that tests for tumour enhancing properties should be included among the requirements for assessing the safety of chemicals (Autrup and Dragsted, in Sweirenega and Yamasaki 1992).

1.1.4 Objectives

This thesis describes the development of a short term *in vitro* assay designed specifically to identify tumour promoters in the skin. In order that the objectives of this proposed system are fully understood, information on the following topics are presented in this introductory chapter:

a) the skin: structure and function (section 1.2),

b) disorders of skin keratinisation (section 1.3),

c) multistage carcinogenesis in the skin (section 1.4),

d) assays for identifying tumour promoters: advantages and disadvantages (section 1.5).

e) detailed aims and objectives of a model of human initiated skin for identifying tumour promoters (section 1.6).

1.2 THE STRUCTURE AND FUNCTION OF THE SKIN

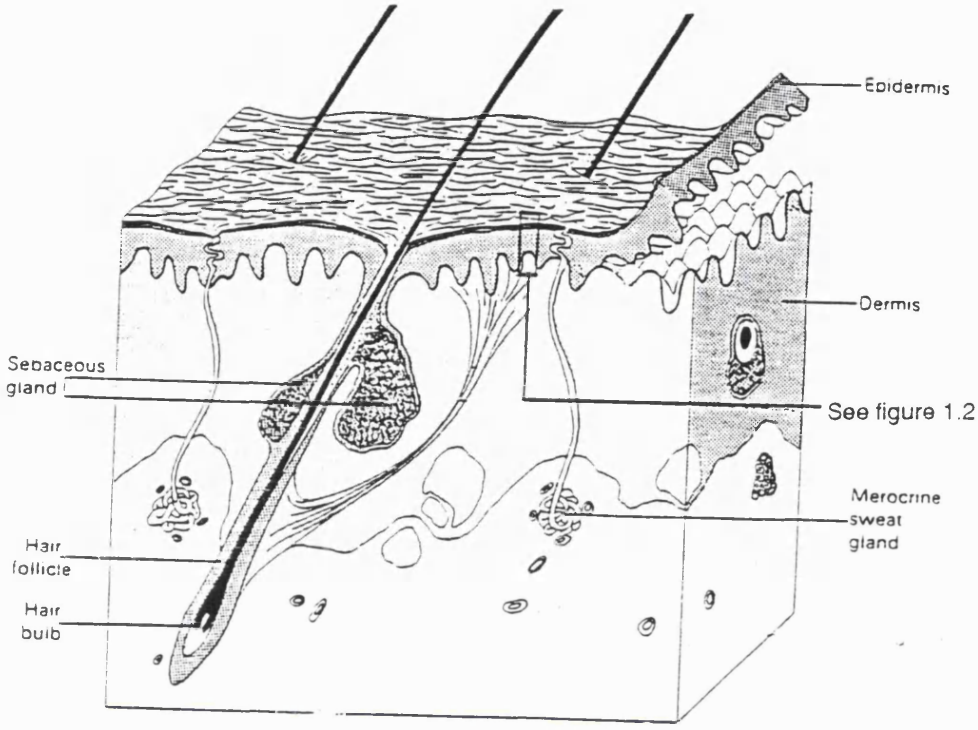
1.2.1 Function

The skin is the tissue that forms the external protective covering of the body of vertebrates. Its main function is to protect the underlying organs against mechanical and chemical injury, bacterial invasion, vapour transmission and also from the destructive effects of ultraviolet rays. It has a sensory function enabling sensations such as pain, temperature and touch. Sweat glands in the skin perform an important role in the excretion of waste products. By dilating and contracting blood vessels the skin is able to regulate body temperature. In sunny climates it is the main source of vitamin D in the body. However the skin is not just an inert protective barrier, it is a dynamic living tissue which is able to perform a number of metabolic and physiological functions.

1.2.2 Structure

The skin consists of two distinct layers: the epidermis, the fine outer layer, and the dermis, the relatively thick underlayer.

Figure 1.1 *The skin*

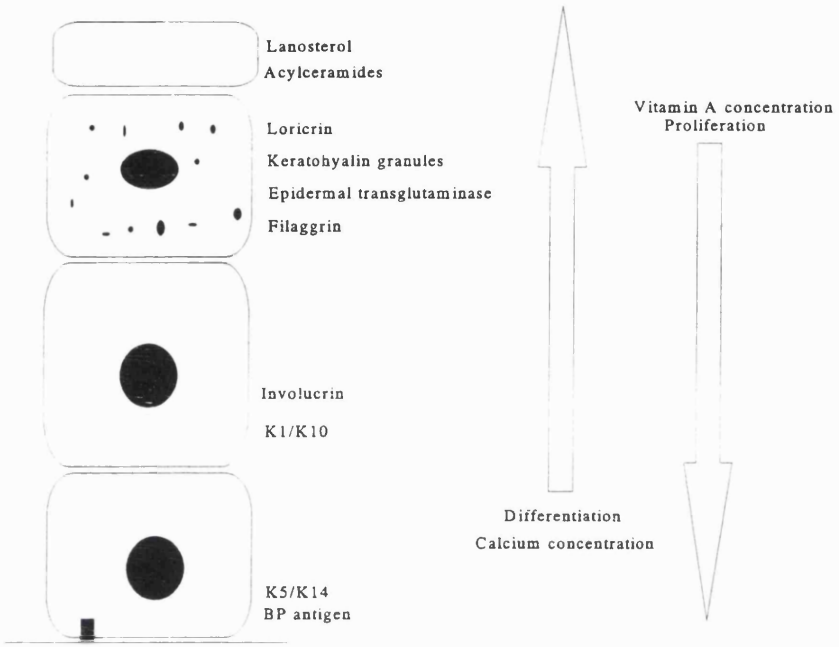


Wheater 1987

1.2.2.1 The epidermis

The epidermis is a stratified squamous epithelium formed from the outer embryonic layer. It varies in thickness from around 1.0 mm on the palms of the hand and soles of the feet, to 0.1 mm on the face. It is made up of four main cell types; melanocytes, merkel cells and langerhans cells, which are involved in pigmentation, sensory and inflammatory responses, respectively. The fourth cell type, keratinocytes, are the most abundant in the epidermis (> 90%) and are responsible for the architecture of the epidermis. The epidermis may be divided into four layers; basal, spinous (or prickle), granular and cornified layers, based on the state of differentiation of the keratinocytes.

Figure 1.2 *The epidermis*



The **basal layer** consists of germinal cells which are actively dividing to produce new epidermis. This single layer of cuboidal cells is separated from the dermis by a basement membrane. The interaction of basal cells with the basement membrane is mediated by the expression of specific integrins, particularly the $\alpha_6\beta_4$ complex, which is polarised in the basal cell surface of basal cells. $\alpha_6\beta_4$ has been reported to co-distribute with the bullous pemphigoid antigen in keratinocytes forming a stable anchoring contact for basal cell adhesion to the basement membrane, and similarities in composition, stability and cell adhesion function have suggested a relation to hemidesmosomes in the skin (Carter 1990). Other integrins e.g.$\alpha_3\beta_1$ mediate cell to cell interactions to maintain the stratified epidermis (Carter 1990, Jones 1991). Basal keratinocytes characteristically synthesise the insoluble polypeptide keratins K5, and K14 and these are involved in cytoskeletal

formation (see section 1.2.3). Although not produced in the suprabasal keratinocytes, these keratins remain in suprabasal cells and are essential for filament formation in the spinous layer. Melanocytes, containing variable amounts of the black pigment melanin which protects the body from the harmful effects of UV rays, are also found in this layer. Infoldings of the basal layer produce sweat glands, sebaceous glands, hair follicles and fingernails. Oil is secreted onto the surface of the epidermis by sebaceous glands which open into hair follicles enhancing waterproofing properties of the skin. Since the epidermis does not have its own supply of blood vessels, germinal cells derive their nutrition and oxygen mainly by diffusion from capillary networks in the dermis. The basal layer is the only germinative layer in the epidermis; suprabasal layers are made up of differentiating cells. As new cells are formed the older ones get pushed outwards toward the surface, flattening as they progress.

When a cell in the basal layer divides it may enlarge and join anabolic keratinocytes in the **spinous cell layer**. Associated with this migration is the loss of proliferative capacity and the suppression of K5 and K14 synthesis. One of the earliest signs of commitment to differentiation is the production of keratins K1 and K10 in the spinous layer (see section 1.2.3). These polypeptides aggregate into bundles in the cytoplasm forming 8 nm intermediate filaments that eventually join with desmosomal plaques in the plasma membrane in the granular layer. Also characteristic of the spinous layer is the production of the soluble protein involucrin (Watt 1989).

The **granular layer** is made up of three to five layers of flattened polygonal cells which

have a granular appearance as a result of keratohyalin (a precursor of keratin) deposits. The production of K1 and K10 is suppressed in this layer whilst loricrin, filaggrin and the enzyme epidermal transglutaminase is enhanced. Activation of this transglutaminase in granular cells results in the cross-linking of the non-keratin proteins involucrin and loricrin to form a permeable, rigid cornified envelope (Watt and Green 1982) that replaces the plasma membrane in cells of the cornified layer. Filaggrin is involved in bundling together keratins to form macromolecules. Cells in the granular layer are only briefly anabolic and soon enter a phase in which cytoplasmic organelles are destroyed (transition zone).

The **cornified layer** is made up of 10-25 (depending upon the part of the body) layers of non-nucleated, flattened, metabolically-inactive keratinocytes devoid of organelles. These cells are packed with keratin filaments and surrounded by an insoluble protein envelope (the cornified envelope) found closely apposed to the inner face of the plasma membrane. Cornified keratinocytes form a protective water resistant barrier and form the first line of defence against micro-organisms, environmental toxins and UV radiation. These cells are continuously shed from the skin and are replaced by upwardly migrating cells from the basal cell layer. Every few weeks approximately 1.6 m^2 of new skin replaces worn surface layers.

A principle regulator of keratinocyte maturation *in vivo* is a gradient of calcium ions that is low in the basal cell compartment and high in the granular cell layer. A vitamin A gradient opposing the calcium gradient is believed to contribute to the regulation of

keratinocyte differentiation (Darmon 1991). It has been reported that the autocrine expression of transforming growth factor (TGF)β_1 restrains basal cell proliferation, whilst TGFβ_2 provides a similar restraint on suprabasal cells (Glick et al 1993). Positive growth control is mediated by TGFα and proliferative effects are mediated by EGF receptors in the basal cells. Another receptor found in the epidermis is the FGF receptor which mediates growth via factors synthesised by cells in the dermal compartment.

Besides its defensive role the epidermis is also able to metabolise xenobiotics using a range of enzymes. Phase I enzymes e.g. cytochrome P450 1A1/2A1, 2B1/B2 and 3A, and epoxide hydrolases, and phase II enzymes e.g. Glutathione-S-transferase and steroid and aryl sulphatases, have all been located in the epidermis (Regnier et al 1990; Mukhtar 1989, 1991; Lillenblum 1986).

1.2.2.2 The dermis

Underlying the epidermis is the dermis. This is a fibrous covering developed from the middle embryonic layer. It varies in thickness from about 0.5-3.0 mm, and provides support and nourishment for the overlying epidermis. The matrix of the dermis is made up of collagen and elastin which are secreted and degraded by fibroblasts. Fibroblasts are also responsible for producing growth factors which stimulate growth and differentiation of keratinocytes e.g. keratinocyte growth factor, whilst keratinocytes release factors which have an inhibitory effect on the growth of fibroblasts e.g. cytokines (Fusenig 1992; Vellucci et al 1995; Rubinchik & Levischaffer 1996). Also present in the dermis are two glycosaminoglycans, which bind large quantities of interstitial fluid and have a role in

mediating the transfer of nutrients and waste products between dermal blood capillaries and the avascular epidermis. Also within the dermis are nerves which enable sensations, and blood vessels which provide nourishment to dermal and epidermal cells. The importance of the dermal component on the epithelial layer can be discerned from experiments in which the epidermis was transplanted onto a different dermal site and took on the phenotype of the epidermis that would normally overlay the dermis (Fusenig 1992).

1.2.3 Keratins

One of the main functions of keratinocytes is to produce keratins. Keratins are a family of water-insoluble polypeptides which characteristically form a large component of the cytoskeleton of all epithelial cells. Currently there are around 30 different keratins of which 20 are epithelial keratins and 10 hair keratins (see review by Smack et al 1994).

The classification of keratins is based on dividing keratins according to molecular weight and pI values on isoelectric focusing. Keratins are separated into type I keratins which have acidic pI values and molecular weights ranging between 40-60 kDa and type II keratins which have basic or neutral pI values and molecular weights ranging between 50-70 kDa (see table 1.1). Each keratin is assigned a number according to its molecular weight so that type I keratins range between K10 and K20 (where K10 is the smallest and K20 the largest) and type II keratins between K1 and K9. Epithelial keratins are co-expressed so that a basic keratin is paired with its equivalent size-ranked acidic keratin, for example, K1 is co-expressed with K10. Generally acidic keratins are 8 kDa smaller

than their basic keratin counterparts.

Table 1.1 Epithelial keratin pairs

Type I Acidic keratins	Molecular weight (kDa)	Type II Basic keratins	Molecular weight (kDa)
K10	56.5	K1	67
K11*		K2*	65
K12	55	K3	64
K13	51	K4	59
K14,K15	50,50'	K5	58
K16	48	K6	56
K17	46	K7	54
K18	45	K8	52
K19	40	K8	52
K20	46	K8	52
		K9	64

* K11 and K2 are breakdown products of K10 and K1 respectively.
Adapted from Smack 1994

Table 1.2 The location of skin keratins.

Keratin pairs	Skin condition	Location
K1 K10	Normal	Suprabasal
K2 K11	Normal	Suprabasal
K4 K13	Malignant tumour	Epidermis
K5 K14	Normal	Basal
K6 K16	Hyperproliferative	Suprabasal
K8 K18	Tumorous	Epidermis

Panlov et al 1994; Smack 1994; Moll 1982

Of the 20 epithelial keratins, only 6 are found in normal skin (see table 1.2). K5 and K14 predominate in the basal keratinocytes. As these cells begin to differentiate and join the spinous layer, translation of messenger RNA encoding K5 and K14 is shut down and synthesis of K1, K2, K10 and K11 is initiated.

The characteristic pattern of keratins synthesised in normal keratinocytes may be altered in disease states, for example psoriasis, epidermolysis bullosa simplex and malignant cancers (see section 1.3.1), and also on application of chemicals such as calcium, phorbol esters and retinoids (see section 1.3.2). Generally K6 and K16 are frequently associated with hyperproliferative skin such as in psoriasis, and also in small quantities in normal epidermis of the soles of the feet. K18 and K13 are present in cancerous skin (Pankov et al 1994; Nischt et al 1988), K13 has been specifically associated with malignant cancers (Yuspa et al 1991).

1.3 DISORDERS OF SKIN KERATINISATION

The regulation and differentiation in the skin is tightly controlled, therefore factors affecting these processes can lead to alterations in normal regulation, resulting in disorders of the skin. The sequential appearance of different keratins during differentiation is believed to be co-ordinated with the migration of the cells towards the surface of the skin. Any alteration in the rate of migration may alter the synthesis and processing of keratins. This may explain the difference in the final size and distribution of the keratins found in some skin diseases.

1.3.1 Skin diseases

1.3.1.1 Epidermolysis Bullosa Simplex (EBS)

EBS is a genetic skin disorder characterised by blistering upon minimal trauma. Initial studies found the presence of keratin filament clumps in the basal keratinocytes prior to blistering (Smack et al 1994). These clumps were composed of abnormal forms of keratins K5 and K14 which contained point mutations that were not present in unaffected family members or healthy controls. Thus mutations of K5 and K14 in EBS were responsible for reducing the mechanical stability of basal keratinocytes. In this disease the dermal-epidermal junction is pathologically altered.

1.3.1.2 Psoriasis

Psoriasis is a disease of the skin in which raised, rough, reddened areas appear. The eruption is associated with alterations in the epidermis which cause a defect in the formation of the cornified layer resulting in an increased production of keratinocytes, and an accelerated rate of keratinocyte maturation and turnover in the suprabasal compartment. The expression of K1 and K10 in the suprabasal keratinocytes is diminished and predominately replaced by K6 and K16. The basal cell layer expression of K5 and K14 is unaffected.

1.3.1.3 Skin Cancer

An altered pattern in keratin expression is seen in both benign tumours and also min carcinomas. The distribution of K5 and K14 in papillomas is similar to that in normal skin whilst levels of K1 and K10 are reduced (Roop et al 1988). Keratin expression in carcinomas is greatly disturbed, K5 and K14 transcripts are diffusely expressed in carcinomas and K1 and K10 are low or diminished (Roop et al 1988). In addition keratins not normally found in skin are expressed in tumours.

K8 and K18 are normally expressed in simple epithelia. The persistent expression of K8 and K18 has been demonstrated in a wide variety of carcinomas derived from epithelia. K18 has been reported to be a direct target of the *ras* signal pathway. Balmain et al (1984) demonstrated that a high proportion of DMBA induced mouse skin carcinomas and papillomas had an activated *ras* gene. Its appearance in papillomas suggest it may have occurred at a relatively early stage of carcinogenesis. Pankov and coworkers (1994)

suggest that activation of Ha-*ras* may stimulate the transcription of K8. Papillomas and carcinomas can be distinguished by the expression of K13. This keratin is normally found in internal stratified epithelia and has also been identified in skin carcinoma but not in normal skin or papillomas (Nischt et al 1988).

1.3.2 Effects of chemicals on keratinocytes

A number of chemicals and hormones are known to influence the rate of proliferation or differentiation of keratinocytes and also therefore affect keratin synthesis.

1.3.2.1 Calcium

A major advance in the culture of keratinocytes came with the discovery that extracellular ionic calcium is a key regulator of epidermal growth and differentiation. The growth rate of normal human keratinocytes (NHK) *in vitro* increases with added extracellular calcium between the concentrations of 0.03 mM and 0.3 mM (Boyce & Ham 1983). Whilst in high calcium medium (>1.0 mM), human keratinocyte growth rate is reduced and terminal differentiation is induced. Thus low levels of calcium may be used to selectively cultivate keratinocytes whereas high levels of calcium can be used to induce stratification and differentiation of keratinocytes. These findings are supported by the calcium gradient in keratinocytes in the epidermis *in vivo* (Watt 1984). A low concentration in basal cells promotes their proliferation whilst increasing concentrations of calcium in suprabasal layers induces terminal differentiation. Calcium has been reported to allow stratification by assisting the formation of desmosomal contacts (Watt et al 1984), and specifically induce type I epidermal transglutaminase, promoting differentiation (Rubin & Rice

1983).

Traditionally cell media have contained high concentrations of calcium due to the presence of calcium in serum (see section 2.2.1). With the finding that enhanced proliferation could be obtained at lower calcium concentrations came commercial low calcium cell media e.g. keratinocyte serum free medium (KSFM Gibco) and MCDB 153 (Clonetics). The benefits of these media are discussed in chapter 2 (section 2.2.2).

The importance of calcium in regulating growth and differentiation has also evoked studies on the effects of calcium signals and diseased states of the skin which involve abnormal maturation of keratinocytes e.g. psoriasis and cancer. During carcinogenesis, initiated cells lose their requirement for low levels of calcium to proliferate and do not respond to internal calcium surges that would lead to cornified envelope formation in normal keratinocytes (Whitfeild 1992). Thus a reluctancy to respond to calcium induced differentiation is an important characteristic of premalignant cells. Epidermal cells from initiated skin and benign or malignant skin tumours and also cells exposed to chemical carcinogens *in vitro* are resistant to the induction of differentiation by calcium *in vitro* (Yuspa 1989)

1.3.2.2 Phorbol esters

12-O-Tetradecanoyl phorbol-13-acetate (TPA) (also known as Phorbol-12-myristate-13-acetate; PMA) is a member of the phorbol ester family. It is found in the seed of the small south east asian tree *Croton tigilium*. TPA is a strong inflammatogenic and

hyperplasiogenic agent in the skin and is a potent tumour promoting agent in experimental animals when applied in combination with a genotoxic carcinogen e.g. Dimethyl benzanthracene (DMBA) (see section 1.4.1). The exposure of keratinocytes to TPA *in vitro* can lead to a multitude of biological and biochemical responses (see table 1.3).

Table 1.3

Cellular responses to TPA	
Increase in	Decrease in
RNA	intercellular gap junction communication
DNA	the retinoic acid receptor*
protein	oestrogen receptor*
ODC activity**	B adrenergic receptor*
polyamine levels**	thymidine incorporation
levels of putrescine	EGF binding
protease activity	activity of catalase^
number of dark cells[a]	activity of superoxide dismutase^
epidermal transglutaminase activity	
differentiation	
activity of protein kinase c^	
production of reactive forms of oxygen	

* Kumar et al 1994, ** O'Brien et al 1975, [a] Klein Szenzo et al 1980, ^Agarawal and Mukhtar 1992 and Slaga 1983

Reports of TPA induced changes in keratinocytes *in vitro* have been contradictory in that both proliferation and differentiation, which are regarded as mutually exclusive events, appear to be occurring simultaneously. For example, factors supporting cellular proliferation such as increases in ornithine decarboxylase (ODC), RNA, DNA and proteins (Slaga 1983) occur at the same time as an increase in epidermal transglutaminase activity (essential for the formation of cornified envelopes (Yuspa et al 1980)). Such conflicting reports have often been attributed to species and cell variations, to differences in culture conditions and TPA concentration.

In 1982 however, Yuspa reported that keratinocytes did in fact respond to the tumour promoter TPA by differentiating and proliferating. He suggested that the basal keratinocytes were the principle target for TPA. In order for both the induction of differentiation and proliferation to occur simultaneously, Yuspa concluded that there must be heterogeneity amongst basal cells and divided them into 'TPA induced differentiation-sensitive' and 'TPA induced differentiation-resistant' cells. When normal basal keratinocytes were exposed to TPA, 90-93% of these cells were induced to differentiate whilst the remainder continued to proliferate (Parkinson 1983). Differentiating cells sloughed from the plate within 1-2 days. The remaining resistant cells were also resistant to calcium (1.2 mm) induced differentiation, and also resistant to differentiation following a second dose of TPA. Instead, a second dose of TPA stimulated the remaining cells to proliferate (Yuspa 1982). If however, the basal cells that did not respond to differentiation following the first exposure to TPA were subsequently cultured for at least 8 days in the absence of TPA, the heterogeneity in response to TPA returned upon re-exposure (Yuspa 1982; Parkinson 1983). Parkinson suggested that the resistant keratinocytes could be in a phase of the cell cycle at the beginning of TPA treatment which enabled them to resist differentiation and thus they continue to proliferate in the presence of TPA. However, when these resistant cells were cultured in the absence of TPA, they were able to mature and take on a TPA-sensitive characteristic.

TPA might stimulate some keratinocytes to differentiate and others (i.e. stem cells and initiated cells) to proliferate, such that repeated doses of TPA would select for initiated

cells by inducing them to proliferate at the expense of the normal cells. In support of this hypothesis cultures of squamous carcinoma cell lines were found to contain a higher percentage of TPA resistant keratinocytes, and mouse epidermal keratinocytes initiated *in vivo* or *in vitro* showed a reduced response to inducers of differentiation.

1.3.2.3 Retinoids

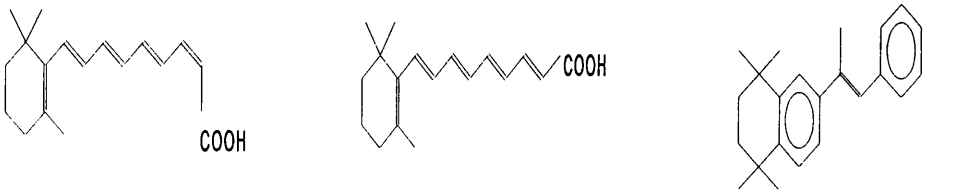
Retinoid is used to describe any compound with vitamin A activity. The effect of vitamin A on epithelial cells was first reported in the 1920's by Mori who demonstrated that a deficiency of vitamin A could induce changes to the epithelia of the respiratory tract. Observations of the effects on the gastro-intestinal and urinary tracts were later described by Wolbach and Howe (1925).

Since then retinoids have been shown to affect epithelial cells of the mammary gland, colon, oesophagus, liver and the skin (see review by Moon and Mehta 1991). Retinoids are required for normal growth and differentiation of epithelial cells (Goodman 1979). Epithelial changes of vitamin A depleted animals include metaplasia and abnormal cornification, desquamation and stratification (Wolbach & Howe 1925).

Much of our understanding of retinoids on the skin has come about through *in vitro* studies. The culture of keratinocytes in vitamin A depleted medium was reported to lead to an increase in the production of the high molecular weight keratins, K1 and K10 (Fuchs & Green 1981) indicating vitamin A may be involved in preventing maturation or enhancing proliferation.

In man vitamin A deficient skin appears extremely dry and hyperkeratotic (Wolbach & Howe 1925). The effect of retinoids on the skin have led to their introduction into clinical medicine, being used to treat patients with previously untreatable keratinization disorders including psoriasis, ichtyoses, Dariers disease and acne.

Figure 1.3 *Synthetic (temarotene) and naturally occurring retinoids*



3-*cis* retinoic acid *all-trans* retinoic acid temarotene (Ro 15-0778)

Animal experiments have demonstrated the usefulness of retinoids in the prevention of cancer of the skin, forestomach, liver, mammary gland and bladder (see Wood 1991). Epidemiologists have shown an inverse relationship between the intake of foods with high vitamin A and a risk of developing cancers. When administered to humans retinods have demonstrated activity in preventing cancers of the skin, head and neck, lungs and bladder (Hill & Grubbs 1992).

However retinoids are also powerful teratogens and their uses are therefore limited. An association between retinoids with a free carboxyl group and all-trans configuration and teratogenic activity has been reported (see Wood 1991). Synthetic analogues of retinoids without these particular features are currently being investigated as potential anti-cancer

agents (see figure 1.3).

1.4 MULTI-STAGE CARCINOGENESIS (MSC)

1.4.1 Introduction

Carcinogenesis is generally accepted as being a multi-step process. Several lines of evidence may be found in Barrett (1987). The concept of MSC was introduced in the 1940's when Berenblume (1941) showed that the sequential application of the polyaromatic hydrocarbon, DMBA and TPA to mouse skin gave rise to tumours. Since then this hypothesis that tumours develop in stages of initiation and promotion has been supported by work in other tissues including the bladder, colon, kidney, liver, lungs and stomach (Pitot 1986).

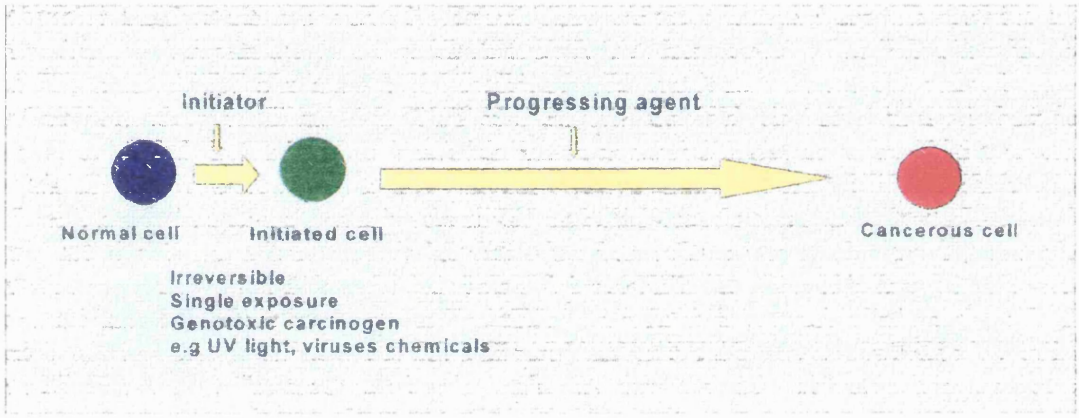
The current understanding of carcinogenesis may be described in 6 stages (Fitzgerald & Yamasaki 1992), however, the most often quoted version involves the stages of initiation, promotion and progression.

1.4.1.1 Genetic events in the MSC theory

Tumour **initiation** is believed to be caused by a single exposure to a genotoxic carcinogen which results in a permanent mutation of a single specific gene. Thus initiators react covalently or indirectly modify cellular macromolecules such as DNA, RNA and proteins. Examples of initiators include UV light, viruses and chemical carcinogens such as polyaromatic hydrocarbons, nitrosamines and aromatic amines (Slaga 1983). The initiated cell persists for the lifetime of the animal despite the fact the epidermis renews itself approximately every 6-8 days (Potten 1983). Initiated cells

express a subtle change in their phenotype which is unrecognisable in intact epidermis. It has been shown in culture these cells exhibit an altered response to signals of differentiation which allows their selective growth under culture conditions favouring differentiation e.g. high levels of calcium and TPA.

Figure 1.4 *Genotoxic events in carcinogenesis*



Under normal conditions the initiated cell lies dormant in its tissue unless it is challenged by an agent which is capable of converting this premalignant cell, via further genetic changes, to a malignant cell. This further genotoxic insult to the initiated cell is termed **progression.** The probability that both events will occur in the same cell (in the absence of other fatal mutations) is extremely small. This acquisition of multiple heritable alterations at independent genetic loci may explain the long latency of cancer.

1.4.1.2 Epigenetic events in the MSC theory

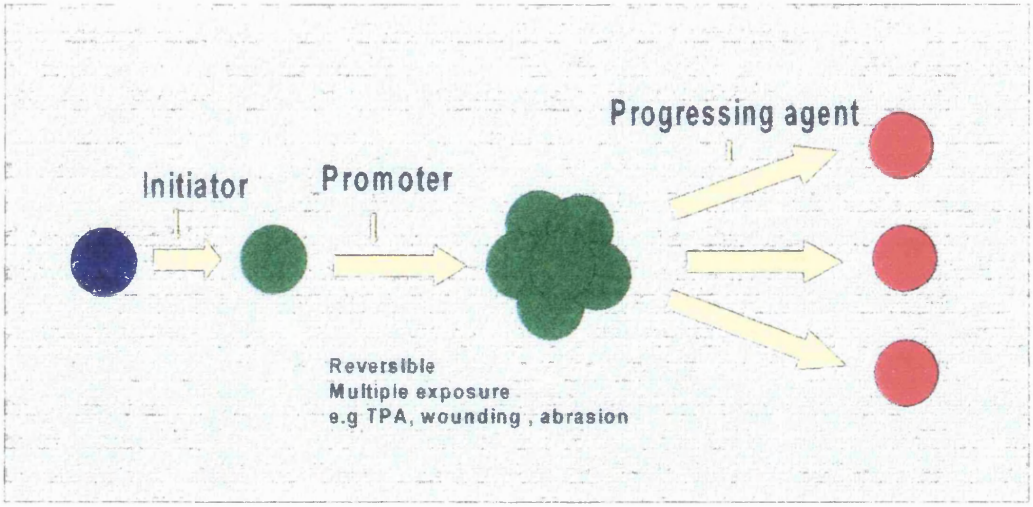
A **tumour promoter** is an agent which is capable of selecting for the growth of initiated cells resulting in their clonal expansion. This expansion of the initiated cell population greatly increases the likelihood of one cell progressing to become a cancerous cell. Tumour promotion requires repeated exposure and, since its effects are reversible, it is believed to be an epigenetic alteration. Promoting agents incorporate a wide variety of compounds including plant products, polycyclic aromatic hydrocarbons, surface active agents, anthrones, organic peroxides, hydroperoxides and long chain fatty acids. Many environmental chemicals are believed

Table 1.4 *Agents reported to promote tumours in order of potency*

Phorbol esters
Teleocidin
Okadaic acid
Tobacco smoke condensate
Benzoyl peroxide
Surface active agents
Citrus oils
Wounding
Abraision
UV light
Silica fibres

Adapted from DiGiovanni (1994)

Figure 1.5 *Epigenetic events in multi-stage carcinogenesis*

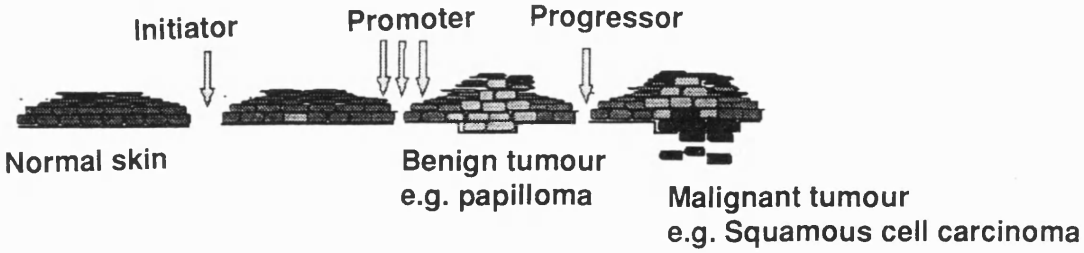


to promote tumour formation e.g. drugs (e.g. oral contraceptives and phenacetin containing analgesics), food additives (e.g. artificial sweeteners) and pollutants (e.g. tobacco smoke and automobile exhaust). By identifying and avoiding exposure to promoting agents it should be possible to reduce the risk of developing cancerous lesions.

1.4.2 Carcinogenesis in the skin

This study is involved in the promotion of basal and squamous cell carcinomas of the skin. Development of such cancers is shown schematically in figure 1.6. Initiation may be induced by a variety of methods but always involves cells in the basal layer since those in the suprabasal layers are committed to differentiate and are therefore unable to proliferate. Initiated skin shows similar morphology to normal skin. Long term application of a tumour promoter may induce clonal expansion of the initiated cell which may give rise to benign tumours. Further application of a genotoxic agent may then induce malignant transformation of one or more of the transformed cells resulting in a squamous or basal cell carcinoma which possesses the ability to metastasise.

Figure 1.6 *Multi- stage Carcinogenesis in the skin*



1.4.2.1 Tumour promotion in the skin

Thus tumour promotion is an epigenetic event resulting in the clonal expansion of initiated cells which in turn leads to the formation of benign tumours. The association of TPA and tumour promotion was identified over 50 years ago, however the mechanism of action by which TPA causes its effects is still largely unknown. Its ability to cause hyperproliferation and inflammation are not sufficient for its tumour promoting ability since other agents with similar properties fail to promote tumours e.g. turpentine and ethylphenyl propionate (Agarwal & Mukhtar 1992). Several effects of TPA on the skin were shown in table 1.3. Some of these changes have been associated with the promoting ability of TPA as they are also shared with other tumour promoting agents e.g. the activation of PKC, induction of dark basal keratinocytes, and of ODC which regulates the synthesis of polyamines.

1.4.2.2 Two stage tumour promotion in the skin

The promotion stage of carcinogenesis can be further divided into stage I (conversion) and stage II (progression) (see review by Slaga 1983). Stage I requires a limited number of applications of a stage I promoter e.g. TPA or TGF β which are not sufficient to produce tumours in initiated skin unless followed by repeated applications of a second stage, incomplete promoter such as mezerein or retinoyl phorbol acetate. Stage II promoters will only promote a tumour following multiple exposures to a stage I promoter. Stage I tumour promotion may be applied before or after initiation. TPA and mezerein appear almost equipotent in ODC induction, and mezerein is also capable of producing most of the morphological and biochemical changes in skin and in cells in

culture as with TPA. However TPA has been reported to be fifty times more active as a tumour promoter. Some of the different responses of TPA and mezerein have been shown in table 1.5. The biochemical basis for the functional differences between complete and second stage tumour promotion remains unresolved.

Table 1.5 *Comparison of cellular and biochemical responses to TPA and mezerein*

	TPA	Mezerein
Induction of epidermal cellular proliferation.	50	100
Stimulation of DNA synthesis.	50	100
Induction of dark basal keratinocytes.	100	25
Tumour promotion.	100	2
Binding to PKC receptor.	100	2

From Slaga 1983.

Further evidence of two stage tumour promotion comes from the finding that certain chemicals are able to selectively block each stage. In 1980 Slaga et al reported that tosyl phenylalanine chloromethyl ketone (TPCK) could selectively block stage I tumour promotion and that retinoic acid showed some specificity for stage II, whilst flucinolone acetonide could block both stages. However in 1992 Agarwal and Mukhtar reported TPCK and FA to inhibit stage I tumour promotion while retinoic acid was reported to block both

Table 1.6 *Selectivity of anti-promoters for stage I and stage II promotion*

Reference	FA	TPCK	RA
Slaga 1980	I+II	I	II
Agarawal & Mukhtar 1992	I	I	I+II

stages (see table 1.6). Although the concept of two stage promotion is generally accepted, the ability of anti-promoters to selectively block either of the stages warrants further investigation.

1.4.2.3 Mechanism of action of tumour promoters

In this study the effects of PKC binding phorbol ester type promoters has been investigated alongside benzoyl peroxide which is a free radical generator (see later this section) and also A23187 which has a mechanism of promoting action involving the ionophores ability to elevate internal calcium concentrations.

Table 1.7 *Mechanism of action of various tumour promoters*

Tumour Promoter	Mechanism of action
TPA Teleocidin Aplysiatoxin	Activation and/or downregulation of PKC Induction of a pro-oxidant state Increase in internal calcium concentration
Okadaic acid	Inhibition of serine threonine phosphatases
Benzoyl Peroxide Chrysobarin	Generation of free radicals leading to a pro-oxidant state (see section 1.4.2.4)
Thapsigargin	Inhibition of calcium activated endoplasmic reticulum ATPases resulting in an elevation of intracellular calcium

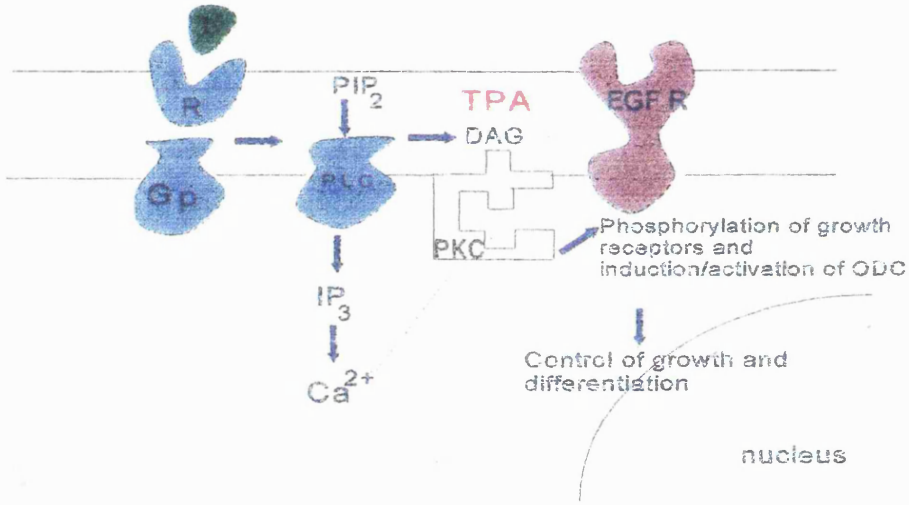
Digiovanni (1994)

PKC activators

It is generally believed that tumour promoting effects of TPA involve binding to PKC (see figure 1.7). TPA activates a calcium dependant isozyme of PKC at the same site as the endogenous activator of PKC diacylglycerol. In its active form PKC is involved in phosphorylating various growth factor receptors leading to the induction of

differentiation in mature keratinocytes and proliferation of initiated and immature keratinocytes (see section on TPA). PKC activity is essential to down regulate K1 and K10 and up regulate loricrin filaggrin and transglutaminase activity during keratinocyte maturation (Dlugosz & Yuspa 1993). Initiated cells are resistant to the terminal differentiation induced by activators of PKC, favouring the growth of these cells which results in the formation of tumours.

Figure 1.7 Mechanism of action of TPA



A schematic depiction of the mechanisms of action of TPA. TPA is believed to induce tumour promotion following activation of PKC. Under normal conditions an external ligand can lead to the activation of PKC by stimulating a signal transduction system which involves the sequential activation of membrane bound receptors (R), G proteins (Gp) and phospholipase C (PLC). Activated PLC breaksdown phosphotydylinositol 4,5-diphosphate (PIP2) into the second messenger inositol 1,4,5-triphosphate (IP3) and DAG. IP3 mobilises calcium from internal stores. The increase in calcium and binding of DAG to PKC activates this enzyme to mediate signals for cellular growth through mechanisms which may involve phosphorylating growth factor receptors (EGF R)and activation and induction of ODC. TPA binds PKC at the same site wher DAG binds to it. Unlike DAG, TPA binding is not transient and therefore induces prolonged PKC activation. ***(See reviews by Berridge 1983, 1984)***

Free radical generators

The tumour promoting activity of peroxides that are chemical sources of free radicals and the ability of antioxidants to inhibit tumour promotion constitute some of the major support for the hypothesis that free radicals are involved in tumour promotion. Free radicals are extremely reactive species which can react with cellular components. Lipid peroxidation is initiated by the attack of a free radical on unsaturated lipids and a resulting chain reaction ensues which ultimately leads to the destruction of the lipid. Damage to lipids in internal cell membranes such as that of the endoplasmic reticulum or mitochondria which sequest calcium, can lead to an increase in cytoplasmic calcium concentration. Amongst the degradation enzymes which are activated by an elevated cytosolic calcium concentration, are phospholipases found in biological membranes, which are involved in catalysing the hydrolysis of membrane phospholipids. Phospholipase A_2 enzymes are involved in detoxifying phospholipid hydroperoxides by releasing fatty acids from peroxidised membranes which may occur during lipid peroxidation. However prolonged activity of these enzymes can lead to the breakdown of the membrane, releasing arachadonic acid. Some of the metabolites of arachadonic acid such as prostaglandins E2 and F2α are important for the induction and maintenance of hyperproliferation in skin. The combination of increased internal calcium concentration and prostaglandins may be responsible for the induction of tumour promotion by such compounds.

1.5 *IN VITRO* ASSAYS FOR IDENTIFYING TUMOUR PROMOTERS

In vitro assays for tumour promoters may be based upon a theoretical understanding of the mechanisms of tumour promotion e.g. inhibition of intracellular gap junctions or the measurement of metabolic profiles of cellular molecules such as ODC or the number of dark cells (see table 1.3). Alternatively, an assay may mimic the events seen *in vivo* which in the case of tumour promotion is the clonal expansion of initiated cells. Both types of assay have been reviewed extensively (see Fitzgerald & Yamasaki 1992; Shaw & Verma 1995).

1.5.1 Mechanistic assays

1.5.1.1 Ornithine decarboxylase (ODC)

ODC plays a key role in the biosynthesis of polyamines which are necessary for growth and differentiation. This enzyme catalyses the first rate limiting step in converting t-ornithine to putrescine. This polyamine is subsequently utilised in the synthesis of DNA.

↑ODC → ↑polyamines → ↑DNA synthesis→ ↑cell proliferation

A large rapid and transient increase in ODC has been reported to be one of the earliest changes that occur following exposure to TPA. The induction of ODC and subsequent rise in intracellular polyamines, have been proposed as obligatory events in tumour promotion (O'Brien 1976). The regulation of ODC is altered as the events of carcinogenesis proceed such that an increase in basal levels of ODC is seen in benign tumours (O'Brien 1976) and this level increases further as tumours progress to

malignancy. Also see articles by Gerhauser et al 1995, and Pegg et al 1994.

The role of an in increased expression of ODC in tumourogenesis was studied by Clifford and co-workers (1995) by constructing a replication-defective retroviral vector toover expresss ODC in epidermal cells. They showed that ODCover expressionn by itself was not sufficient to induce tumours in normal cells but that increased ODC enhances tumour development in initiated premalignant cells.

Since enhanced ODC activity appears to be induced by a range of tumour promoters including those that do not activate PKC, (Suganuma et al 1981; van Rooijen et al 1987), and agents that are able to reduce ODC levels e.g Difluoromethyl ornithine (DFMO) (Weeks et al 1982; Takigawa et al 1983) also have anti promoter activity, whilst known anti-promoters such as retinoic acid (Verma & Boutwell 1977) and inhibitors of prostaglandin synthesis have also been reported to reduce ODC levels (Verma et al 1980), induction of ODC has frequently been used to screen potential promoters (Gimez-Conti et al 1991).

However when the magnitude of ODC inducability was compared with susceptibility to tumour promotion in three strains of mouse (C57Bl/6, DBA1L and SENCAR), ODC induction was reported to be independent of tumour susceptibility (Imamato 1992). Furthermore *in vivo* studies show TPA to be a far stronger promoter than mezerein however both compounds induce equipotent levels of ODC (Slaga 1983). Thus although capable of identifying promoters levels ODC do not appear to reflect the potency of

promoters or susceptibility to promotion.

1.5.1.2 Gap junctional intercellular communication (GJIC)

Of all *in vitro* assays for tumour promoters, inhibition of intercellular gap junctions has probably received the most attention (see review by Yamasaki 1990). Gap junctions are specialised intercellular channels between apposing plasma membranes that allow the transfer of low molecular weight hydrophilic molecules. By facilitating the transfer of second messengers such as cyclic AMP, calcium ions and inositol triphosphate, gap junctions play an important role in cellular differentiation and proliferation.

Since the process of cancer involves a loss in growth control, the disease has been associated with intracellular communication. Abnormalities in gap junctions have been observed in cancer cells in cell number as well as structural and functional defects (Ljungquist and Warngard 1990). Initiated cells which are scattered randomly in the population of normal cells remain dormant for long periods of time under the growth regulating influences of their normal neighbouring cells. The blockage of GJIC may result in the modulation of repressor effects of normal cells on adjacent initiated cells leading to their clonal expansion. There are four lines of evidence supporting the importance of cellular communication in tumour promotion:

(i) several agents capable of inducing tumour promotion *in vivo* also alter cellular communication in *in vitro* studies (Yotti et al 1979; Murray & Fitzgerald 1979; Zeilmaker and Yamasaki 1986).

(ii) oncogene products have been reported to inhibit GJIC (Fitzgerald & Yamasaki 1992).

(iii) the inhibition of GJIC was reported in a TPA sensitive cell line whilst no inhibition was seen in a TPA resistant cell line (Rivedale 1985).

(iv) the inhibition of GJIC by promoters can be suppressed by anti-promoting agents such as retinoic acid cyclic AMP and glucocorticoids (Willecke 1991; Yamasaki and Katoh 1988).

These findings have encouraged the development of assays based on the inhibition of GJIC for identifying tumour promoters and anti promoters (Fitzgerald & Yamasaki 1992). However although inhibition of GJIC measured *in vitro* shows some correlation with tumour promoting activity, Kam (1988) showed that phorbol ester tumour promoters were not able to inhibit GJIC of mouse epidermal cells *in vivo.* Further there are many types of tumour promoter, with diverse mechanisms of action, and not all appear to affect GJIC e.g. non-phorbol ester type tumour promoters such as okadaic acid, TGFβ and TCDD failed to inhibit GJIC (Hamel et al 1988; Boreiko et al 1986; Abernathy et al 1985).

1.5.2 Cell transformation assays

End points which have not been proved to be directly related to tumour production e.g. GJIC or changes in metabolic profiles are likely to give false positive and negative results. Clonal expansion assays were developed as extensions of cell transformation assays.

Cell transformation occupies a unique position among short term carcinogen tests since this type of assay is capable of detecting genotoxic and non-genotoxic activity of chemicals and therefore allows the identification of both initiators and promoters.

1.5.2.1 The assessment of initiators in cell transformation assays

Reiznikoff et al (1973a) reported of transformation studies using cells derived from embryos of C3H Heston strain mouse in which a dose dependant increase in the number of morphologically transformed foci were observed after exposure to several polycyclic aromatic hydrocarbon carcinogens. Cells isolated from these foci were shown to induce fibro sarcomas after injection into syngeneic mice. Whereas injections of up to 10 fold greater numbers of non-transformed parental cells did not produce tumours (Reiznikoff et al 1973b). Thus a genotoxic chemical carcinogen could be identified, *in vitro*, by its ability to transform a normal cell. Transformation was not only seen with chemicals but also following exposure of cells to viruses and UV radiation (see Stoker 1966).

1.5.2.2 Assessment of tumour promoters in cell transformation assays (clonal expansion)

1.5.2.2.1 Clonal expansion assays utilising fibroblasts

Exposure of fibroblasts to a low concentration of a chemical carcinogen caused some of the cells to become initiated. In 1966, Stoker reported the growth suppression of transformed cells when they were co-cultured with normal cells. He showed that contact of polyoma virus transformed hamster fibroblasts with normal hamster or mouse fibroblasts inhibited the growth of transformed cell foci. Thus when positioned among a monolayer of normal cells these initiated cells did not clonally expand (see figure 1.8).

Later it was shown that treatment with phorbol esters enhanced the growth of SV40 transformed (Sivak & Van Duuren 1967) and *myc* oncogene transfected (Bignami et al 1988) 3T3 fibroblasts when they were co-cultured with normal 3T3 cells, and also UV light induced C3H10T1/2 transformants when co-cultured with normal C3H10T1-2 cells (Herschmann & Brankow 1987).

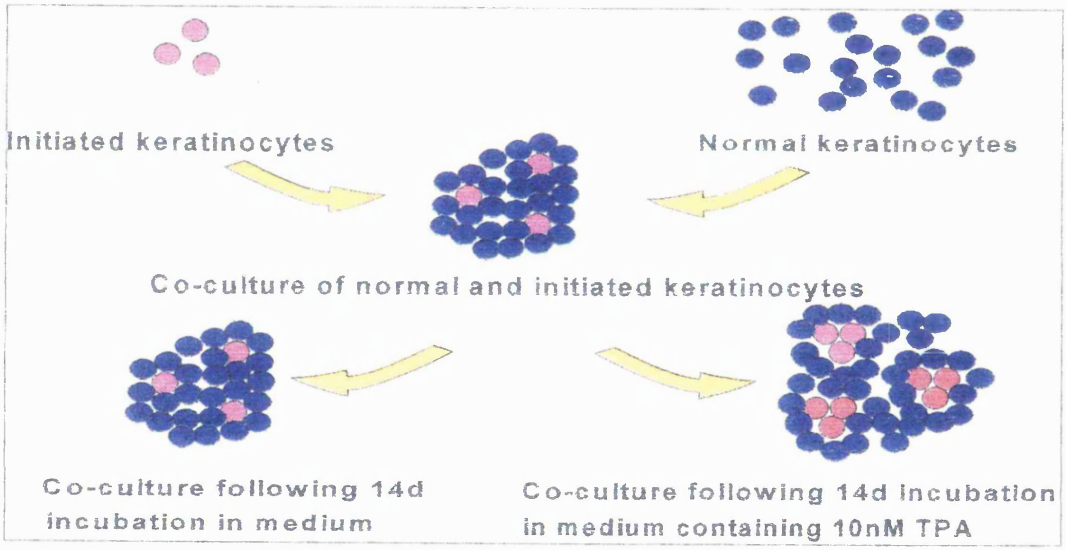
The fibroblast clonal expansion assays for identifying tumour promoters were able to identify some but not all classes of promoters. Firstly very few tumours arise from fibroblasts. Because of their regenerative nature epithelial cells are frequently the sites for malignant transformation *in vivo*, consequently most tumours are of epithelial origin. Also, tumour promoters are tissue specific e.g. phenobarbital TPA and oestrogen are promoters in the liver, skin and breast respectively. Clearly an *in vitro* assay would have to employ a specific cell type to assess tumour promotion in a particular tissue e.g. hepatocytes or keratinocytes for liver or skin tumour promoters respectively.

1.5.2.2.2 Clonal expansion assays utilising keratinocytes

Henry Hennings and co-workers in 1990 reported on the development of an *in vitro* analogue of initiated mouse epidermis to study tumour promoters and anti-promoters. Based on the fibroblast co-cultures they set up a clonal expansion assay utilising mouse keratinocytes to model initiated mouse epidermis. Initiated cells (cell line 308) were derived from calcium resistant foci of keratinocytes from adult BALB/c mouse skin which had been initiated *in vivo* with DMBA.

Co-culture of a small number of these cells with confluent normal primary keratinocytes resulted in the inhibition of growth of colonies of 308 keratinocytes. This growth suppression was inhibited on addition of tumour promoters to the co-cultures resulting in the clonal expansion of colonies of 308 cells (see figure 1.8). Furthermore simultaneous treatment with promoters and anti-promoters was reported to inhibit clonal expansion induced by promoters. This system is discussed in more detail in chapter 3.

Figure 1.8 *A valid model of initiated epidermis*



Clonal expansion of initiated cells should be inhibited by surrounding normal cells and this inhibition overcome following exposure to tumour promoters.

Initial results from this assay, though limited, suggest excellent concordance with *in vivo* mouse data. However such an assay may not be useful for identifying tumour promoters in human skin. Not only is there a need to compensate for species differences but there is also a need for *in vitro* to *in vivo* extrapolation.

1.5.3 Tumour promoter assays: the problem of species variation

Differences in species response to multi-stage carcinogenesis has been varied. Mouse skin appears to respond better to initiation -promotion protocols of carcinogenesis compared with rats and hamsters. Even within species there are differences in response to the induction of papillomas by treatment with DMBA and TPA. There is still no conclusive evidence to support multi-stage carcinogenesis in human skin. Classification of compounds as tumour promoting agents has essentially been achieved through rodent bioassays. Information from these studies is then used to predict human responses without sufficient knowledge of similarities or differences between the species. Recent studies comparing human and rodent cells *in vitro* have implicated important differences in enzymes which may play a crucial role in carcinogenesis, suggesting that rodent data may not be applicable to humans.

1.5.3.1 Species variation in PKC isozymes

TPA involves the activation of PKC in order to induce tumour promotion in mouse keratinocytes. If there are quantitative or qualitative differences between human and mouse keratinocyte PKC isozymes, as has been reported (Fischer et al 1993) (see table 1.8) then both the *in vivo* mouse skin assay and Hennings *in vitro* model of initiated mouse skin may be inadequate for studying tumour promoter activity in humans.

Table 1.8 *Isozymes of PKC from freshly isolated keratinocytes*

	Present	Absent
Freshly isolated mouse keratinocyte	α β δ ζ	γ ϵ
freshly isolated human keratinocytes	α β δ	γ ϵ ζ

Fischer et al 1993.

1.5.3.2 Species variation in ODC induction

A comparison of ODC activity following TPA treatment of mouse and human keratinocytes demonstrated that like *in vivo*, mouse keratinocytes *in vitro* showed an increase in ODC activity following exposure to TPA, however no induction of ODC activity was seen after exposing human keratinocytes to TPA *in vitro*. These findings were confirmed when levels of ODC mRNA of both cell types matched levels of the ODC protein (Fischer et al 1993).

1.5.3.3 Species variation in glutathione-S-transferase isozymes

Glutathione-S-transferases (GST) are a family of widely distributed phase II metabolism detoxification enzymes that catalyse the reaction between glutathione and various hydrophobic electrophiles including free radicals (see section 1.4.2.4). Since free radical generation has been associated with both TPA and non TPA type promoters, glutathione and GST may therefore have a role in antagonising tumour promotion.

Species specificity in GST isozymes have been reported under normal and altered physiological conditions (Raza 1991). Three classics isozymes have been reported representing products of distinct genes GST alpha (basic), GST mu (neutral), and GST

pi (acidic). Human skin was found to contain the alpha isozyme of GST, which is absent in rats and mice, whilst both rodent species produce GST mu, which is absent in human keratinocytes (see table 1.9).

Table 1.9 *Species variations in GSH isozymes in skin.*

Species	Alpha	Mu	Pi
Rat	✗	✓	✓
Mouse	✗	✓	✓
Human	✓	✗	✓

Adapted from Raza 1991

Thus species variation in enzymes which have been implicated in tumour promotion present the possibility for species differences between rodents and humans in multi-stage carcinogenesis.

1.5.4 Tumour promotion assays: the problem of *in vivo* to *in vitro* extrapolation

1.5.4.1 Fibroblasts

In 1983, Coulomb and co-workers showed that fibroblasts grown in monolayer differed from those *in vivo* in three respects. Diaminobenzidine (DAB) is able to penetrate across the plasma membrane of fibroblasts *in vivo*, however fibroblasts in monolayer are not permeable to DAB whilst they are attached to plastic, or following detachment by mechanical scraping. Collagen processing appears to differ depending upon the way in which fibroblasts are cultured. *In vivo* fibroblasts synthesise collagen which is then released to form the matrix of the dermis. However, when grown in monolayer, although fibroblasts are able to synthesise collagen, most of it remains soluble in the medium and only a small percentage is completely processed to form a matrix (Nusegens et al 1984). Normal fibroblasts *in vivo* express peroxidases associated with the nuclear envelope and

endoplasmic reticulum. No such activity was detected in fibroblasts grown in monolayer.

1.5.4.2 Keratinocytes

Keratinocytes also deviate from normal when cultured in submerged monolayer. Cellular retinoic acid binding protein (CRABP) is an example of a protein which appears to require epidermal stratification and/or epidermal-dermal interaction. Two forms of CRABP (CRABP I and CRABP II) have been characterised in murine, rat, bovine, avian and human skin. Recently Astrom and co-workers (1991) reported the selective induction of CRABP II in human skin following topical application of retinoids *in vivo* and also by agents which induce keratinocytes *in vitro*. These data suggest that CRABP II may function to regulate the cutaneous actions of retinoids in epidermal differentiation. However when cultures of keratinocytes in submerged monolayer were exposed to retinoids *in vitro* no increase in the levels of CRABP II were detected.

Thus keratinocytes and fibroblasts which are the major cell types of the skin deviate from normal behaviour in culture. Steps have been taken to improve culture techniques so that cells are cultured in an environment which resembles their situation *in vivo*. Rather than culturing single cell types, attempts have been made to culture groups of cells from a tissue in a similar topography to their situation *in vivo* in organotypic cultures. Thus human organotypic cell cultures may provide the solution to the problems of species differences (section 1.5.3) and *in vitro in vivo* extrapolation (section 1.5.4).

1.5.5 Organotypic culture

The last decade has shown an increasing interest in the reconstitution of various epithelial cell perhaps the most successful being the reconstituted model of skin. The reconstituted model of skin is made up of two components: a solid dermal substrate which is most usually made up of type I collagen and dermal fibroblasts; and an epidermal component made up of differentiating keratinocytes which is cultivated on the dermal substrate. This skin equivalent sits at an air-liquid interface such that the dermal component is immersed in medium but the epidermal component is exposed to the air as the situation *in vivo*.

It has been shown that fibroblasts in the skin equivalent resemble those *in vivo* more closely than those in submerged monolayer in that 80% of these fibroblasts express the perinuclear and endoplasmic reticulum peroxidases. Fibroblasts in the skin equivalent are also able to penetrate DAB and a large proportion of the collagen synthesised by these fibroblasts in the skin equivalent is fully processed and added to the matrix of the structure (Nusegens 1984). In a similar way keratinocytes on the skin equivalent, unlike those cultured in submerged monolayer, are able to synthesise CRABP II following exposure to retinoic acid as is seen *in vivo*.

1.6 AIMS

The co-culture model of initiated mouse epidermis presented by Hennings appears to have the potential to identify tumour promoters and anti-promoters. A major setback of this model is the use of mouse keratinocytes. If as the preliminary data suggest, mouse *in vitro* and *in vivo* data correlate, one might assume that a similar model of initiated epidermis using human keratinocytes could be useful in determining promoter activity in humans, avoiding species differences. However *in vitro* to *in vivo* differences still exist. By culturing the cells in an environment similar to the situation *in vivo* i.e. in organotypic culture, it should be possible to improve the ability of these cells to retain *in vivo* like characteristics. For example, although Hennings co-culture has been called a model of initiated epidermis in fact only one layer of keratinocytes are present and this does not represent a stratified epithelium. In this study an attempt was made to set up a skin equivalent containing normal and initiated human keratinocytes to form a model of initiated human epidermis.

Firstly, a model of normal human skin was developed by amalgamating the methods of Asselinaeu (1986) and Lenoir (1988). The skin was reconstructed using only readily available sources so that it could be easily set up on a regular basis in any tissue culture laboratory (chapter 2). Co-cultures of normal and pre-neoplastic human keratinocytes were set up using a similar method to Hennings mouse co-cultures, and the effects of promoters and anti-promoters were assessed (chapters 3 and 4). Finally the co-culture was incorporated within the skin equivalent to form a model of initiated human epidermis (chapter 5).

CHAPTER TWO

The Development of a Normal Skin Equivalent

2.1 INTRODUCTION

Human skin differs from animal skin in a number of ways including in thickness, hair and gland density and metabolising capacity. Thus the unique characteristics of human skin limit, to a great extent, the use of animal models for evaluating new drugs and the toxicity testing of new products. These limitations have encouraged the development of several *in vitro* models of human epidermis. Advantages of using these models include a reduction in the number of living animals used, and the ability to study the effects of toxicants on human cells.

2.1.1 *In vitro* submerged culture of keratinocytes

Normal interfollicular epidermal cells are usually derived from foreskin or mammary reductions and grown in submerged monolayer in either serum-containing or serum-free media.

2.1.1.1 Serum-containing media

In order for cells to survive and grow in culture, they need to be placed in a medium which can provide all the environmental conditions that the cells were exposed to *in vivo*. Serum is the major biological fluid supporting survival and growth of mammalian cells *in vivo*. In addition to carbohydrates, proteins, minerals, calcium and vitamins, it also contains many hormones and growth factors.

Early systems for culturing keratinocytes involved removing the epidermis from the dermis using trypsin (Medawar 1941). Pure cultures of the epidermal cells were then

seeded at high density in serum supplemented medium (Cruickshank 1960). Confluent layers were achieved after 2-3 days and their life expectancy was around 2-3 weeks. This system required large amounts of skin since lower seedings of epidermal cells failed to grow.

The system was greatly improved when Rheinwald and Green (1975a) reported the ability to grow pure cultures of keratinocytes from a single low density suspension of epidermal cells using a feeder layer of X-irradiated mouse 3T3 fibroblasts in serum-containing medium. This technique was based on the knowledge that *in vivo*, keratinocytes are dependant on the presence of fibroblast in the dermis for their supply of growth factors. A simple co-culture of fibroblasts and keratinocytes results in fibroblast proliferation in the exclusion of the slower growing keratinocytes. To avoid this problem fibroblasts were growth inhibited by irradiation or with the use of alkylating agents such as mitomycin c. Although unable to proliferate, these growth arrested fibroblasts were still capable of secreting essential growth factors. The lifetime of keratinocytes was greatly improved as they could be passaged through multiple generations. Without feeder fibroblasts keratinocytes have a 100-1000 fold reduced ability to form colonies in cloning experiments (Smola 1994). Recently it has been reported that dermal micro-vascular endothelial cells are as effective as fibroblasts at stimulating epidermal cell proliferation (Smola 1993). Keratinocytes grown in serum-containing medium in the presence of irradiated fibroblasts could be cultivated for around 50 cell generations and this was subsequently improved by the addition of various growth factors including epidermal growth factor (EGF) which increased keratinocyte lifetime in cultures to around 150

generations (Rheinwald & Green 1977).

2.1.1.2 Serum-free media

Although initially believed to be essential for keratinocyte culture the presence of serum was associated with experimental variability. Uncontrolled conditions due to undefined components in the serum, and batch to batch variation evoked the need for a more defined culture medium. Also, primary keratinocytes derived from skin often contain small amounts of fibroblasts from the dermis. Thus culturing primary keratinocytes by the Rheinwald and Green method frequently gave rise to fibroblast overgrowth due to stimulation of contaminating fibroblasts by TGFβ released from platelets during the preparation of serum.

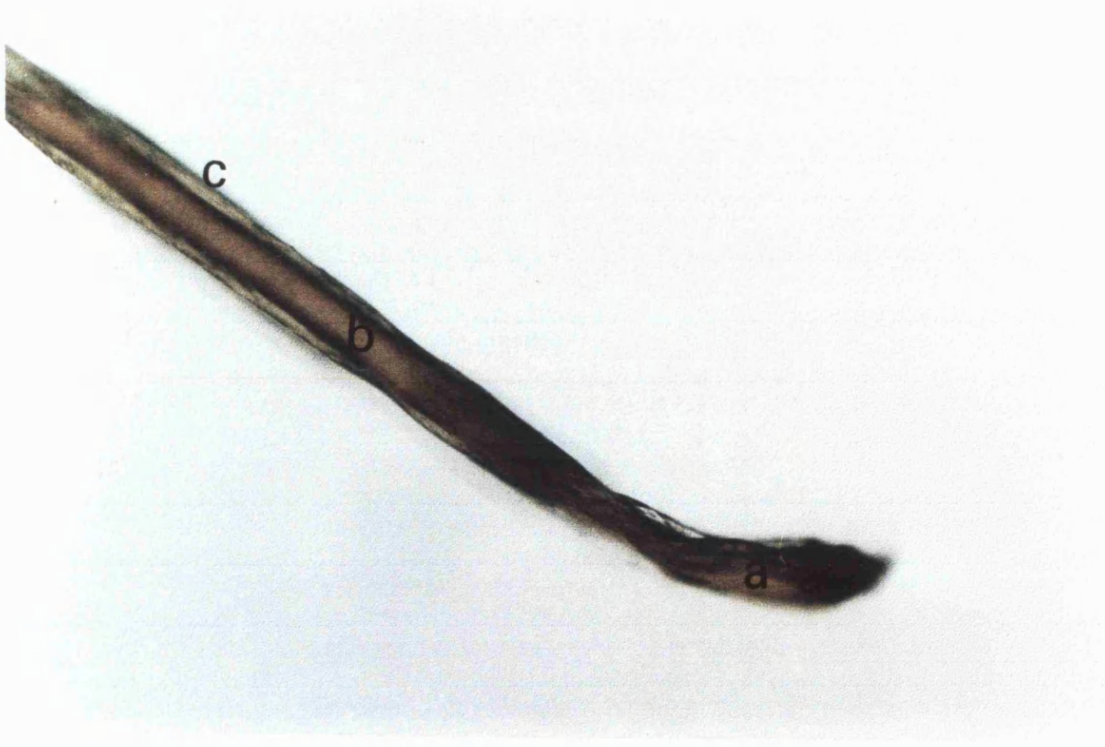
In an attempt to improve reproducibility and standardisation between laboratories, in 1983, Boyce and Ham introduced a defined serum-free medium (MCDB 153) for the selective culture of keratinocytes in the absence of feeder layers. This medium contained a very low concentration of calcium which improved proliferation of keratinocytes and reduced terminal differentiation resulting in a better survival of cells following sub-culture (see section on calcium 1.5.1). Since the introduction of MCDB 153, a range of serum- free media have been developed for commercial use. When grown in these media the keratinocytes fail to form desmosomal interconnections (Hennings 1980) and are spaced out as a monolayer with a characteristic "cobblestone" appearance. Although the keratinocytes fail to stratify, they commence terminal differentiation with the expression of involucrin when calcium levels are restored (0.6 mM) (Watt 1984). Compared to

serum containing media, serum-free systems are heavily weighted towards proliferation.

2.1.1.3 Keratinocytes derived from hair follicles

The problem in obtaining a regular source of skin lead to the culture of keratinocytes from plucked hair follicles. Keratinocytes from this source have been successfully cultured on bovine lens capsul (Vermorken 1985), as explants (Wells 1982) and as a single cell suspension on fibroblast feeder layers (Limat & Noser 1986), on plastic.

Figure 2.1 *Outer root sheath of a human hair follicle*



a, hair bulb;b hair follicle; c outer root sheath

The outer root sheath of hair follicles in the anagen phase are surrounded by a layer of rapidly dividing keratinocytes. These cells may be enzymatically detached from the hair sheath and cultivated on plastic using similar techniques to those employed for keratinocytes derived from skin. Although this appears to present only a few keratinocytes, unlike keratinocytes from skin, almost all keratinocytes derived from the hair follicle have the capacity to divide. One single hair follicle can produce around 10^6 cells before cultures reach senescence. Although developed for culturing keratinocytes derived from skin, both the serum containing, and serum-free techniques are also useful in growing keratinocytes derived from the outer root sheath of hair follicles.

2.1.1.4 Deficiencies in monolayer cultures

The submerged culture of keratinocytes has proved to be useful in understanding factors influencing differentiation and proliferation of keratinocytes such as TGF β, calcium and vitamin A, and also the individual roles of keratinocytes and fibroblasts *in vivo* (discussed in chapter 1). However, submerged cultures have been designed specifically to favour propagation and expansion of cells but lack any structural properties of the tissue *in vivo*. Deficiencies in growth and differentiation characteristics of keratinocytes have been attributed to the unnatural environment and the absence of mesenchymal influences found *in vivo* (Hennings & Holbrook 1983). Since these cells have undergone numerous doublings in non-physiological conditions they may be far removed from their *in situ* state. They show a reduced ability to form an even, multi-layered epidermis and as a result contain little if any of the markers of differentiation, characteristic of a normal functioning epithelium (see table 2.2).

2.1.2 Organotypic culture of the skin

Keratinocytes grown on non-biological substrates and submerged under culture medium display poor differentiation which hinders their use for studying epidermal physiology. Organotypic systems attempt to culture keratinocytes in a physiological environment. These culture systems make use of mobile biological matrices as dermal substrates (see table 2.1) upon which keratinocytes from various sources may be attached.

Table 2.1

Substrates used to grow keratinocytes at the air liquid interface.	Reference
Inert filters	Rosdy & Clauss 1990
Nylon mesh	Slivka & Zeigler 1993
Fibroblast-collagen	Bell et al 1979
De epidermised dermis	Regnier et al 1981

The resulting skin equivalent (SE) is placed at an air-liquid interface. This creates a more natural environment and has been shown to be a necessary condition for the initiation and continual synthesis of epidermal differentiation markers (Asselineau et al 1986). Enhanced epidermal development at the air-liquid interface is believed to be due to the establishment of chemical gradients in the epidermis as a result of water loss in the more superficial layers. The SE is able to mimic the normal structure of the skin and unlike cultures in monolayer all four strata of the epidermis and a range of markers of differentiation including keratins 1 and 10, filaggrin, both rigid and fragile types of cornified envelope and fatty acids are present in SE models (see table 2.2 for details).

Table 2.2 *A comparison of the presence of markers of differentiation in keratinocytes grown in submerged and air-liquid cultures*

	In vivo	*In vitro* submerged culture	*In vitro* air-liquid culture
Strata: Basal Spinosum Granulosum Corneum	 ✓ ✓ ✓ ✓	monolayer of basal cells with little stratification	 ✓ ✓ ✓ ✓
Intact basement membrane	✓	✗	✓almost continuous
Hemidesmosomes	✓	ND	✓
Desmosomes	✓	ND	✓spinous layer
Keratohyalin granules	✓	✗*	✓granular layer
Membrane coating granules	✓	ND	✓granular layer
Cornified envelope	✓	low* fragile type only	✓fragile and rigid
Physical barrier to xenobiotics		N/A	10 times more permeable than skin
Keratins	K5/14 K1/10	K5/14 K6/16	K5/14 K1/10 K6/16
BP antigen	✓	ND	✓ polarised
Filaggrin	✓ granular layer	✗*	✓granular layer
Involucrin	✓spinous layer^	✓*	✓ granular layer
Transglutaminase	✓	✓*	✓granular layer

Adapted from Shaw 1995; *Regner 1990; ^Watt 1989; Rosdy & Clauss 1990; Coulomb 1986, 1984; Lenoir et al 1988. ND not done, N/A not applicable

A further advantage of these organotypic models is that test compounds may be applied systemically i.e. in the medium or as in the case of many environmental toxicants topically, by direct application on the epidermal surface.

Such organotypic cultures not only allow the study of normal cell differentiation and pharmacological and toxicological response to drugs, but also allow the ability to reproduce pathological conditions to test these drugs. Physiological effects that have been monitored using SEs include absorption (Roguet et al 1994), phototoxicity (Cohen et al 1994), release of inflammatory mediators (Slivka & Zeigler 1993) and metabolic capacity (Pendlington et al 1994; Slivka et al 1993) .

2.1.2.1 Dermal component

A dermal substrate needs to be porous to allow the diffusion of agents through the dermis, allowing upward diffusion of nutrients in the culture medium to reach basal cells of the epidermal component and downward penetration of topically applied test compounds. The substrate also needs to be transportable so that once formed, it can be lifted easily and placed at an air-liquid interface. Some dermal substrates e.g. de-epidermised dermis, utilise samples of real dermis to recreate an environment similar to which the normal human keratinocytes (NHK) originated. Whilst others e.g. inert poly carbonate membranes, show no resemblance to the dermal component *in vivo,* however they are able to create a dermis using readily available materials. The advantages and disadvantages of three types of commonly used dermal equivalents (DE) will be discussed.

2.1.2.1.1 De-epidermised dermis

Human skin obtained from a skin bank is de-epidermised; the slit is above the basal lamina which is still organised. Collagen type IV, laminin and proteoglycans are still present. Dermal cells are killed by successive freezings and thawings. These substrates can be stored frozen until use, at which stage suspended human keratinocytes can be seeded on the epidermal side of the dermis. After attachment, the de-epidermised dermis bearing the epidermal cells is lifted onto a grid so that the keratinocytes are exposed to the air (Regneir et al 1981).

After 7 days, classical cellular and differentiation markers are correctly located. NHK cultured on a de-epidermised dermis have been reported to stimulate a fully differentiated epidermis which contains the presence and correct location of a whole range of differentiation markers, ultra structural resemblance to skin, and a continuous basement membrane. Furthermore this type of culture has been reported to react physiologically to natural and synthetic hormones such as retinoids and vitamin D (Regnier et al 1990). Also, normal and abnormal keratinocytes (malignant and transformed cells) have been successfully cultured on de-epidermised dermis to monitor their response to pharmacological agents. The barrier function of this type of SE was however reported to be diminished compared with normal skin, but comparable to incubated skin (Roguet 1994).

This type of substrate is largely dependant upon a skin bank and donor variation is

unavoidable. Further, the dermal cells which have been reported to play an integral part in epidermal responses are dead.

2.1.2.1.2 Inert filter substrates

In order to avoid using an undefined dermal component, such as the de-epidermised dermis, Rosdy and Clauss (1990) generated a well differentiated epidermis *in vitro* by growing keratinocytes in a defined medium on inert filter substrates (cellulose acetate or polycarbonate membranes). Again the architecture of resulting SE was very similar to that of normal epidermis *in vivo*, and the presence and correct location of various markers of differentiation (keratins, filaggrin, involucrin and epidermal transglutaminase) was reported. Ultrastructural similarities were seen in the presence of abundant desmosomes in the spinous layers and keratin filaments in cornified layers. Also, the presence of significant amounts of specific epidermal lipids lanosterol and ceramides were present in cornified layers. However no continuous basement membrane could be observed and only keratinocytes at passage two were able to form a fully differentiated epithelium, no cornified layers were observed in this model when first or third passage keratinocytes were used.

2.1.2.1.3 Fibroblast collagen lattices

Collagen (Lillie et al 1980) and fibroblasts (Rheinwald & Green 1975b) both promote the growth of keratinocytes *in vitro*. In 1979 Eugene Bell demonstrated that a collagen lattice seeded with fibroblasts contracted into a tissue and that the phenomenon was dependant upon the concentration of cells and protein. This tissue like structure which resembled

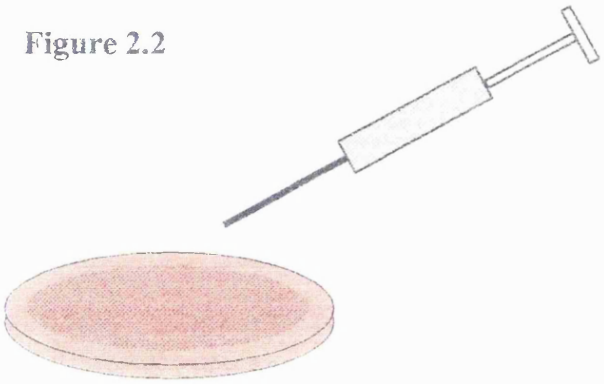
the dermis both in its appearance and its constituents and was the impetus for extensive research into the development of a three dimensional reconstruction of an epidermis on this type of substrate.

The collagen fibroblast lattice has major advantages over both de-epidermised dermis and inert filter substrates. The fibroblast collagen lattice is able to grow keratinocytes at various passages unlike the inert filter model developed by Rosdy and Clauss, and unlike the de-epidermised dermis it is made up of readily availible materials. Further, both de-epidermised dermis and inert filter substrates utilise keratinocyte suspensions to recreate an epidermis. The collagen fibroblast lattice may also be seeded with suspended keratinocytes (Asselinaeu 1984,1986), but in addition an epidermis may be recreated by implanting skin (punch biopsy) (Coulomb et al 1986), or hair follicle (Lenoir et al 1988) explants.

2.1.2.2.1 Creating an epidermis: Seeding with suspended keratinocytes

Early passaged keratinocytes are seeded onto the lattice inside stainless steel rings (Asselineau 1986). The culture is first kept submerged for 1 week to allow the epidermal cells to form a confluent monolayer of basal cells. The cultures are then elevated to the air-liquid interface on stainless steel grids. The cultures are grown for 1 more week and receive nutrients from the medium

Figure 2.2

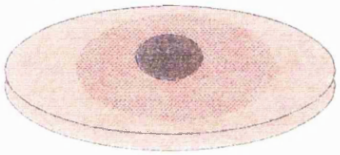


via diffusion through the collagen lattice. Differentiation is near normal as determined by morphological, immunohistological and biochemical methods.

2.1.2.2.2 Creating an epidermis: Punch biopsies

Punch biopsies (3mm) are rinsed with culture medium and placed at the centre of a freshly cast collagen gel containing fibroblasts (Coulomb 1986). The epidermal-dermal junction is positioned at the surface of the gel. During the next 4-5 days the gel contracts around the biopsy, holding it in place. Once contracted the gel resembles a tissue and is raised to the air-liquid interface of the medium. A new epidermis emerges centrifugally from the biopsy displaying morphological and biochemical differentiation markers similar to those found *in vivo*.

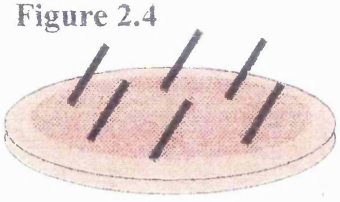
Figure 2.3



2.1.2.2.3 Creating an epidermis: Hair follicles

The outer root sheath of human hair follicles are implanted in freshly cast dermal equivalents (Lenoir et al 1988). After contraction of the lattice the follicles are held upright and positioned at the air liquid interface. Implanted hairs are removed after 8-10 days of culture. A reconstituted epidermis forms in less than a month of culture. Within 10-12 days 1 cm^2 of lattice can be covered by epithelial outgrowth. The epidermis displays extensive differentiation. This system relies on a non-invasive and potentially inexhaustible supply of keratinocytes, and replaces the need for animal and human skin.

Figure 2.4



2.1.3 A comparative study of source of keratinocytes cultured on collagen fibroblast lattice

Having opted to adopt the collagen fibroblast lattice for cultivating cells at the air-liquid interface, the best source of keratinocytes needed to be assessed. A comparison of SEs produced from either hair follicle explants, skin biopsy explants or dissociated keratinocytes derived from either fresh skin or hair follicles had already been reported by Lenoir and Bernard (1990). Their findings indicated that all SEs were able to synthesise a multi-layered epidermis containing a stratum basal, spinosum, granulosm and corneum and produce a basement membrane at the epidermal-dermal junction. The presence of markers of differentiation filaggrin, keratohyalin granules and cornified envelopes were reported to be correctly located just beneath the cornified layer of all SE tested. Also present in all types of SE were desmosomes, fatty acids and the bullous pemphigoid antigen. SE were distinguished by the homogeneity of the constructed epidermis, the shape of basal cells and the expression of the 67 kDa keratin.

Both types of explant (skin biopsy and hair follicle) were favoured over dissociated keratinocytes since basal cells in the SE's derived from explants were cuboidal, as skin *in vivo*, whereas basal keratinocytes on SE derived from dissociated cells were reported to be flattened. Of the two types of explants, SE derived from hair follicles explants produced a homogeneous tissue with uniform expression of keratin 10 in supra basal layers whilst SE derived from skin biopsies tended to be thicker around the biopsy and like dissociated cells, expression of K10 in supra basal layers was delayed and patchy. A summary of these results is shown in table 2.3.

Thus generally SE on collagen-fibroblast lattices are very similar morphologically and in the presence of biochemical markers. However differences documented in Lenoirs study indicated that hair follicles may be the best source of keratinocytes in order to produce an even epithelium with cuboidal basal cells and homogenous location of the 67 kDa keratin marker in suprabasal cells.

Table 2.3

HFE	SB	IF NHK	ORS NHK
----------Multi layered epidermis obtained with overall architecture similar to normal human epidermis----------			
cuboidal shaped basal cells as in normal human epidermis		Basal cells did not have typical cuboidal shape	
Reconstructed tissue homogenous	Reconstructed tissue consistently thicker near biopsy	----------Reconstructed tissue homogenous----------	
----------Collagen type iv deposited linearly at the dermal -epidermal junction----------			
----------Filaggrin and keratohyalin correctly located just beneath cornified layers----------			
Supra basal homogenous expression of 67 kDa keratin	----------Expression of 67 kDa marker delayed and patchy----------		

Adapted from Lenoir and Bernard 1990. HFE hair follicle explants, SB skin biopsy, IF NHK interfollicular normal human keratinocytes, ORS NHK outer root sheath normal human keratinocytes.

It is essential to recognise that all the SEs discussed lack a lot of the biochemical and cellular components of the epidermis (e.g. langerhans and merkel cells) and dermis (e.g. circulatory and nervous tissue) and that they contain no epidermal appendages. Organotypic cultures display a vastly improved epidermalisation as compared to conventional cultures (see table 2.2). The cells tend to form a more differentiated epidermis with stratum corneum and granulosm and keratohyalin and membrane coating granules, and the synthesis of lipid constituents (Lenoir et al 1988; Rosdy & Clauss 1990), essential for maintaining barrier functions, is close to complete.

The interest in organotypic cultures of skin led to the production of many commercial sources of SEs. Although beneficial in that characterised SE could be used to monitor a whole variety of toxicological and pharmacological effects using developed protocols and thus to some extent provided inter laboratory consistency, the cost of commercial SE was excessive and as a result some of the original commercial preparations are no longer on the market.

The purpose of this study was to recreate a three dimensional model of initiated skin which may be used specifically to study the mechanisms of action of tumour promoters. This chapter describes preliminary studies in which a normal SE was reconstituted. In designing the most appropriate model for this purpose, particular attention was paid to reproducibility and availability. Since the model was to be used as an assay, it needed to be highly reproducible, and be composed of materials that were readily available so that it could be set up in any laboratory. In addition it should be possible to incorporate

initiated cells within the normal epidermis to form a model of initiated epidermis (chapter 5). Finally since the cancer process is associated with abnormal growth and differentiation, significant importance was placed upon the ability of the resulting SE to form a fully differentiated epidermis exhibiting as close to normal a pattern of maturation as in skin *in vivo* as possible.

2.1.4 A novel skin model using readily available sources

In initial studies we amalgamated two well studied models (Asselineau et al 1986 and Lenoir et al 1988) to produce a simplified reconstruction of human skin using readily available sources, namely keratinocytes derived from the outer root sheath of hair follicles, and a DE comprising GM10 human embryonic fibroblasts and collagen derived from rat tail collagen and grown in medium containing foetal calf serum (Verma & Shaw 1994).

Table 2.4 *Methods for reconstituting skin*

	Keratinocytes	Fibroblasts	Serum	Collagen
Verma & Shaw 1994	Hair follicle explant*	GM10 human cell line	FCS	Rat tail
Lenoir 1988	Hair follicle explant	Human primary	Human AB	Rat tail
Asselineau 1986	Epidermal keratinocytes in suspension	GM10 human cell line	FCS	Calf Skin

* *In later studies suspended keratinocytes derived from hair follicles were substituted for hair follicle explants*

The DE was formed *in vitro* by mixing collagen, fibroblasts and tissue culture medium. Type 1 collagen is available commercially but can be quite expensive. Rat tail collagen can be easily produced using a cost effective method (Bell 1979). The use of a human embryonic fibroblast cell line (GM10) rather than primary fibroblasts improves the lifetime of cultures since they can be cultivated for longer periods of time. Also foetal calf serum was used in the culture medium rather than human AB serum, as no advantage of using the latter had been documented. Further the former is less expensive and more readily available. This mixture formed a gel in the culture vessel within a few minutes.

2.1.5 Methods for characterising the skin equivalent

Ideally the reconstructed epidermis should exhibit the anatomic and physiological properties of normal skin. Extensive studies of the different types of SE have already been carried out with regard to histology, ultra structure, enzyme expression, release of cytokines and growth factors. Although the modified SE is an amalgamation of two well characterised models it still needs to be characterised in similar detail. Since this model of normal epidermis is to be developed further by incorporating initiated cells into the epidermis, characterisation at this stage was limited to histology and keratin production and location on the premise that if keratins are correctly identified and located and the SE appears to form all four layers of a fully differentiated epidermis histologically, then it is also likely to also show characteristics demonstrated in other normal SE. More detailed characterisation such as the presence and location of markers of differentiation, enzymes and ultrastructural details of this normal SE will need to be carried out simultaneously with the finished model of initiated epidermis so that factors that are altered in

preneoplastic skin e.g. ODC levels, can be compared. Thus preliminary studies characterising the normal SE was restricted to histological staining and the identification and location of skin keratins.

2.1.5.1 Histology

Growth of keratinocytes on SE were characterised histologically to assess stratification and cornification.

2.1.5.2 Keratin synthesis

The ability of keratinocytes on the SE to produce the correct pattern of keratins was assessed using the technique of SDS-page and Western blotting and compared with keratin production of normal skin and NHK grown in monolayer. The capacity of these keratinocytes to differentiate was determined by the presence and correct location of K10 by immunofluorescence.

2.2 METHODS

Materials see Appendix (i)

Culture media see Appendix (ii)

2.2.1 Preparation of a keratinocyte monolayer

A mitomycin c treated 3T3 fibroblast feeder layer was prepared before isolating NHK from hair follicles. Mitomycin c is normally packaged in 2 mg aliquots, and is stable at room temperature for up to 1 month. Since it was required at a concentration of 1 mg/ml, and 7 µl /75 cm^2 flask, it was economical to prepare a large batch of fibroblasts at any one time. Confluent monolayers of 3T3 fibroblasts were exposed to 0.7 µl/ml for two hours then washed thoroughly in PBS, 5 x 1 minute washes. Cells were then incubated in Dulbeccos Minimal Essential Medium (DMEM) 24 hours prior to seeding keratinocytes, or frozen down in liquid nitrogen. Mitomycin treated fibroblasts last for 3-4 days before they detach from the flask at which stage a fresh layer of cells was added.

2.2.2 Isolation of NHK from hair follicles

Hair follicles were plucked from young volunteers all aged between 18 and 30. Only hair follicles in the anagen phase were used, these were identified by a white sheath located just above the bulb of the hair follicle (see figure 2.1). In general these were most prevalent in those volunteers with thick hair.

The outer root sheath was collected in sterile Hair follicle medium and stored in the incubator until sufficient hair follicles were obtained (approx 6-8 hair follicles per 10

cm^2). The follicles were never stored for more than two hours. After removing the hair bulb and excess hair, the outer root sheaths were washed twice in PBS to remove any calcium (from hair follicle medium) and the follicles were incubated in 2 ml trypsin-EDTA for 30 minutes with vigourous pipetting every 10 minutes. Trypsin breaks down the bonds holding the keratinocytes together on the follicle. This reaction was inhibited by the addition of 8 ml DMEM. The cell suspension was centrifuged at 800 rpm for 5 minutes. The supernatant was then removed and the pellet resuspended in 1 ml Rheinwalds medium with choleratoxin (RM+C).

The resuspended cells were added to a flask containing a mitomycin c treated 3T3 feeder layer and sufficient RM+C added to the flask (9 ml /75 cm^2). These cells were refed every other day with RM+C. Amphotericin may be added to the medium at this stage, however this causes the 3T3 cells to detach at a faster rate. Mitomycin c treated fibroblasts were added every week until colonies of keratinocytes of approximately 200 cells were obtained. Approximately 2 weeks of culture were required before colonies of keratinocytes became confluent.

Human epidermal cells grown in RM+C formed tightly adherent epitheloid colonies which by the time they had attained a size of 50-200 cells, had begun to stratify. Stratified cells could be seen under a dissecting microscope yellow in colour, originating from the centre of the colony. It was necessary to subculture these cells before, or as soon as differentiated cells appeared in order to enable them to withstand subculture. Generally these cells were not allowed to grow, in any one flask, for more than two weeks. Cells

were used before they reached passage 4 as this was found to be the life time of primary keratinocytes in culture.

2.2.3 Preparation of a DE

Sterile NaOH 0.1 M, 2 x EMEM, collagen 2 mg/ml, fibroblasts (GM10) 4 X 10^5/ml and foetal calf serum were prepared then added into a petri dish, in order:

Table 2.5 *Constituents of a DE*

	Volumes per 60 mm dish	Volume per 90 mm dish
EMEM	4.6 ml	11.2 ml
FCS	0.9 ml	2.2 ml
NaOH	0.5 ml	1.2 ml
Collagen	3.0 ml	7.3 ml
Fibroblasts	1.0 ml	2.4 ml

The contents of each dish were mixed well immediately and placed in an incubator at 37°C. After 7 days culture immersed in medium the SEs were raised to the air-liquid interface.

2.2.4 Schedule for seeding cell suspension onto DE

Day 1 DE were prepared as described.

Day 5 Medium was replaced with SE medium. Stainless steel rings were placed on the DE, so that DE was seated on the base of the petri dish. 3 - 5 x 10^5 cells in 0.5 ml medium were then seeded into the ring. After a minimum of three hours, the ring was removed and cells cultured submerged for one week changing the medium twice.

Day 12 DE were lifted to the air-liquid interface by placing them on stainless steel grids and then cultured for 14 days* in SE medium, changing medium twice weekly.

Day 28 Histology / Addition of test agent.

* Asselineau (1986) cultured SE for 7 days at the air-liquid interface, in this study insufficient cornified envelope formation at this stage resulted in an extension of the time cultured at the air-liquid interface to 2 weeks.

2.2.5 Schedule for implanting hair follicle explants into DE

Day 1 Prepared DE were incubated for one hour, prior to implanting hair explants into the collagen gel.

Day 5 Contracted lattices were placed on a steel grid at the air-liquid interface and medium replaced with SE medium.

Day 10 Hair implants were removed.

Day 28 Histology / Addition of test agents

Rather than implanting hair follicles into the gel immediately (Lenoir 1988), the gel was allowed to set for one hour prior to implantation as this improved the ease by which hair follicles could be correctly positioned so that the outer root sheath of the hair follicle was positioned half way into the DE and upright mimicking its natural topography in skin. The gel immersed in medium was contracted by the fibroblasts over a few days to form a firm and dense structure around the hair follicles.

2.2.6 Histology

Dermal equivalents were fixed in formalsaline then incubated at 37°C for 30 minutes in 0.01% nile blue sulphate. Epidermal outgrowth stained blue could be visualised in rinsed samples.

2.2.7 SDS page electrophoresis

See Appendix (iii) for buffers.

2.2.7.1 Extraction of cytoskeletal proteins

Skin samples were stored frozen at -80°C. When required the epithelium from the frozen skin was removed mechanically with a razor blade and placed in Buffer 1. Whole SE was placed in buffer 1 (DE alone was used as a control).

2.2.7.2 Preparation of cytoskeletal proteins for electrophoresis

Samples in Buffer 1 were minced and homogenised and then centrifuged at 10,000g for 10 minutes at 4°C. The supernatant was discarded and the pellet resuspended in Buffer

2 and disrupted with a sonicating probe, 3 bursts for 15 seconds each (at a level just before the solution foams), to remove keratins from living layers. This mixture was heated to 95°C for 5 minutes, centrifuged as before, then resolubilised in buffer 3 to extract keratins from cornified layers and finally mixed 1:1 with Laemmli buffer and boiled for 3-5 minutes before adding to the gel. Once mixed with Laemmli buffer the samples may be stored in the freezer for up to 1 year.

2.2.7.3 Preparation of the polyacrylamide gel.

The running gel was prepared by mixing the solutions shown in table 2.6 in sequence. The gel was poured into gel cuvette 1 cm below the top. This was then overlayed with water to help provide a flat interface upon setting. Once set, the water was carefully removed, the stacking gel mixture poured to the height of the cuvette and a well forming comb inserted. As the stacking gel set the comb was removed and samples and marker proteins added to the wells.

Table 2.6 Constituents of polycrylamide gels

Solutions	Separating gel (10%) (ml)	Stacking gel (4%) (ml)
Bis acrylamide solution	2.5	0.666
Tris HCl 1.5 M	2.5	-
Tris HCl 0.5 M pH 6.8	-	1.25
Distilled H_2O	5	3
10% Ammonium persulphate	0.05	0.05
10% SDS	0.1	0.05
TEMED	0.005	0.01

The gel was run at 20 mA until the front marked by the bromo phenol blue tracking dye reached the stacking gel/running gel interface at which point the current was increased to 40 mA and continued until the gel reached 0.5 cm from the gel margin. The gel was carefully removed from the cuvette a corner cut off to mark its orientation and was then allowed to equilibrate in transfer buffer for 30 minutes.

2.2.8 Western Blotting

Nitrocellulose membrane and twelve sheets of Whatman 3 MM paper were cut to the size of the gel and presoaked in transfer buffer. The gel was recovered onto a glass plate and a sheet of soaked 3 MM paper placed on the gel. Any air bubbles were removed by rolling a pipette over the surface. In a similar way five more sheets of 3 MM paper were placed on the first. The whole assembly was then inverted and the presoaked nitrocellulose paper placed on the other side of the gel followed by the remaining six sheets of 3 MM paper. The sandwich was then placed into the electrophoresis tank and filled with transfer buffer and run at constant voltage 11V for one hour. After blotting the nitrocellulose was soaked in storage buffer overnight at 4°C.

2.2.9 Immunohistochemistry

2.2.9.1 Preparation of skin and skin equivalent samples

All samples were washed thoroughly in PBS (3 x 2 minutes). Skin samples were cut to 5 mm^3 whilst SEs were processed whole. Samples were fixed in 4% formalsaline overnight at room temperature, then washed again in PBS, then dehydrated as follows:

70% methanol	2 minutes
100% methanol	2 x 2 minutes
100% histoclear	2 x 2 minutes

Dehydrated samples were then embedded in paraffin wax, sectioned to 7μm and mounted onto silanised glass slides. These slides were dewaxed by rehydrating (procedure reverse of dehydrating) prior to immunostaining. The rehydrating process partially restores blocked antigens which are formed as proteins experience altered conformation during dehydration and waxing.

2.2.9.2 Preparation of cells

Cells were washed thoroughly in PBS then fixed with either formal-saline, for haematoxylin and anti-keratin staining, or with methanol and acetone (1:1) when staining for the large T antigen (chapter 5).

2.2.9.3 Staining procedure

Specimens were incubated in 5% normal goat serum (NGS) diluted in 0.5% dried milk in PBS for 20 minutes. Following removal of the NGS sufficient primary antibody was added to samples:

100 ul added to microscopic slides and 12 well plates

500 ul added to wells of 6 well plate

1 ml added to nitrocellulose blots.

Samples were incubated in the primary antibody for one hour upon which unbound antibody was tapped off and the slides washed in PBS 3 x 2 minutes. The secondary antibody was applied in a similar manner to the primary and also washed 3 x 2 min. Dried slides conjugated with the fluroscein antibody were mounted with hydromount whilst peroxidase stained slides and culture plates were mounted with glycerin after incubating in diaminobenzidine (DAB) solution for 10-60 seconds. All nitrocellulose blots were conjugated with the peroxidase antibody and exposed to DAB solution for 1 minute then air dried and stored at room temperature in foil.

Table 2.7 Antibody types and dilutions

Antibody	Antibody type	Identifies	Dilution
AE3	Primary	Type II keratins	1:100
AE2	Primary	K1/10/11	1:20
K8.60	Primary	K10/11	1:50
M20	Primary	K8	1:100
K8.12	Primary	K13/15/16	1:100
416	Primary	large T protein	1:1
Fluroscein conjugate (FITC)	Secondary	Primary AB	1:100
Peroxidase conjugate	Secondary	Primary AB	1:200

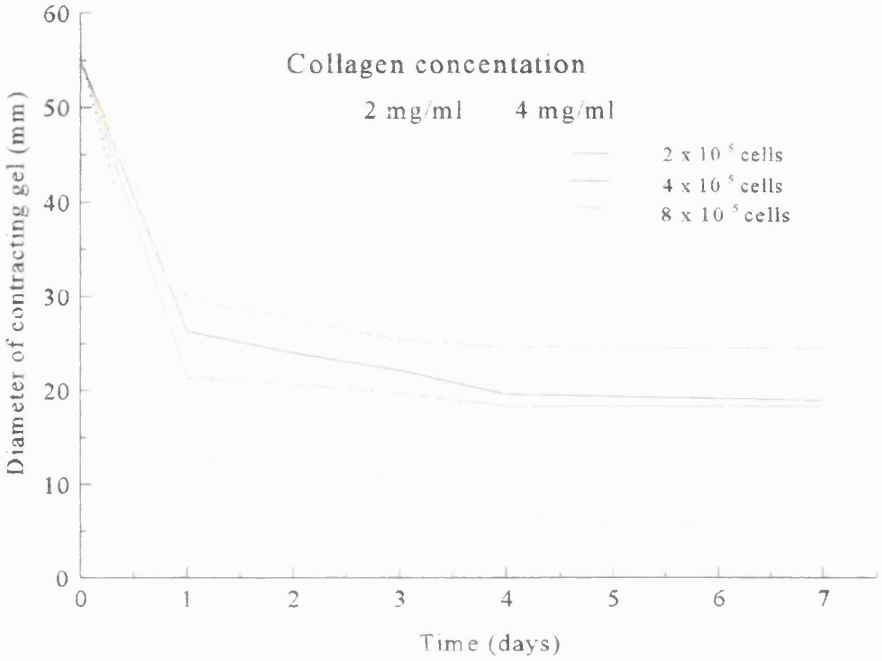
2.3 RESULTS AND DISCUSSION

2.3.1 Contractility of the DE

A DE was formed *in vitro* by mixing together collagen, fibroblasts and tissue culture medium. Optimal conditions for gel contraction were reported to be 4 x 10^5 fibroblasts in 6 mg type I collagen (Coulomb et al 1986). This mixture formed a transparent gel which was the same shape as the culture vessel, within a few minutes. These DE were approximately 13 mm in diameter and 1-2 mm in thickness. DE were highly reproducible both within and between experiments. Most of the contraction was seen within the first 24 hours, after which the rate of contraction was significantly less (see figure 2.5). The speed of collagen contraction was increased by increasing the number of living fibroblasts and decreased by increasing collagen concentration. The size of the gels could also be controlled by varying the amount of fibroblast and collagen concentration (see figure 2.5).

Fungal infections proved to be a problem in initial studies and thus the effects of Amphotericin B on contraction of the DE was assessed. Although Amphotericin B had adverse effects on 3T3 growth it did not appear to affect GM10 growth on plastic, nor did it affect the rate of contraction of collagen-fibroblast gels and was therefore added to medium in subsequent experiments.

Figure 2.5 *Graph to show the effect of collagen concentration on the rate of contraction of the dermal equivalent*



In the absence of fibroblasts there was no gel contraction.
Data are means of experiments repeated at least 3 times.

2.3.2 Growth of hair follicle explant derived keratinocytes on a dermal equivalent

Figures 2.6a-d show the growth of keratinocytes derived from hair follicle explants on a DE over a period of 28 days. After 7 days immersion in medium, keratinocytes from hair follicles had proliferated and formed a monolayer of basal cells on the DE (figure 2.6a). Raising the keratinocytes to the air-liquid interface induced them to begin to form a multi-layered epithelium (figure 2.6b) which differentiated to produce a cornified layer by day 21 (figure 2.6c). SE have been reported to have been maintained for at least 60 days (Lenoir et al 1988). For the purpose of this study, SE need to survive 42 days (28

day growth period plus 14 day exposure to test agent). Figure 2.6e shows a SE at day 42.

Upon staining batches of SE with either nile blue sulphate (whole sample) or haematoxylin and eosin (sectioned sample), it became evident that there was variation in the amount of epidermalisation between samples and within samples. Some SE had little or no epithelial layer whilst others formed 20-25 layers within 21 days. Also, regions of high proliferation and high differentiation were observed within SE. Such inconsistencies in epidermal thickness on the SE had not been reported in previous publications, however other scientists within the laboratory, and also independent work at another laboratory (Astra Charnwood -personal communication) occasionally found the absence of, or poorly formed epithelium on the SE that had been derived from hair follicle explants.

In some cases the process of histology caused the separation of the dermal and epidermal layers so that some samples lost part, or all, of their epithelium. The separation of dermal and epidermal components suggested a relatively poor connection at the junction of SE since this was rarely seen with samples of human skin. Cornified layers were also seen to be detached from other suprabasal layers but this was seen in normal skin samples.

More often the poorly formed epithelial layer was not a histological artefact. Staining with nile blue sulphate entails very little handling of samples and thus absence of an epithelial layer in these samples was unlikely to have resulted from mechanical damage. The variation between the amount of epidermalisation between samples was attributed to the donor variation in hair samples. It was impossible to predict how well any

particular hair follicle in the anagen phase would proliferate on the DE.

Overall the reproducibility of this technique is questionable as epithelial growth can vary between donors of explants e.g. a hair follicle from a donor with thick hair and a large outer root sheath will often produce more keratinocytes than a donor with fine hair and perhaps a thinner outer root sheath. If there are variations in the quantity of proliferating keratinocytes cultured on each DE then one would expect a variation in the size of the resulting epithelia.

In contradiction to the study by Lenoir and Bernard (1990), which reported the formation of a homogeneous epithelium with hair follicle explants, the low reproducibility of this system made it undesirable for use in an assay or as an *in vitro* model for studying the mechanisms of skin disorders. As a result, hair follicle explants as a source of keratinocytes was abandoned. Subsequent experiments utilised keratinocytes which were still derived from hair follicles, but which were first dissociated then grown in submerged culture until they reached passage 3, at which point 5×10^5 cells were seeded onto DE in a manner similar to that described by Asselineau (1986) using keratinocytes derived from skin.

Figure 2.6 *Growth of an epithelial layer derived from hair follicle explants cultivated on a collagen fibroblast lattice at the air-liquid interface.*

Figure 2.6a *Day 7*

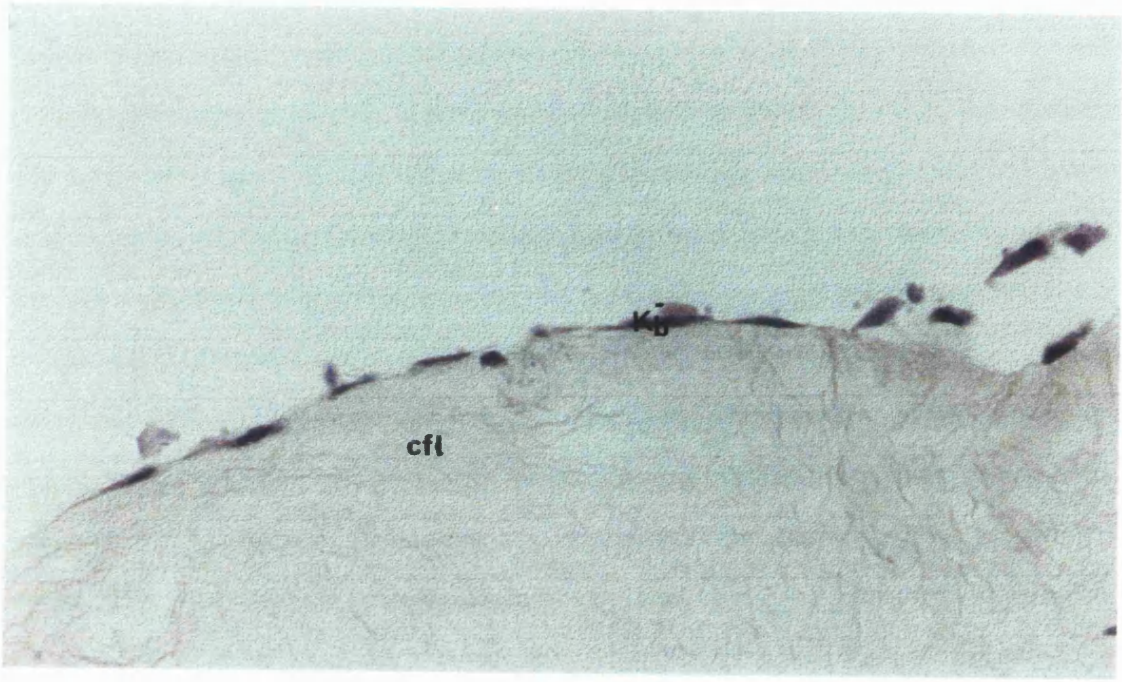


Figure 2.6b *Day 15*

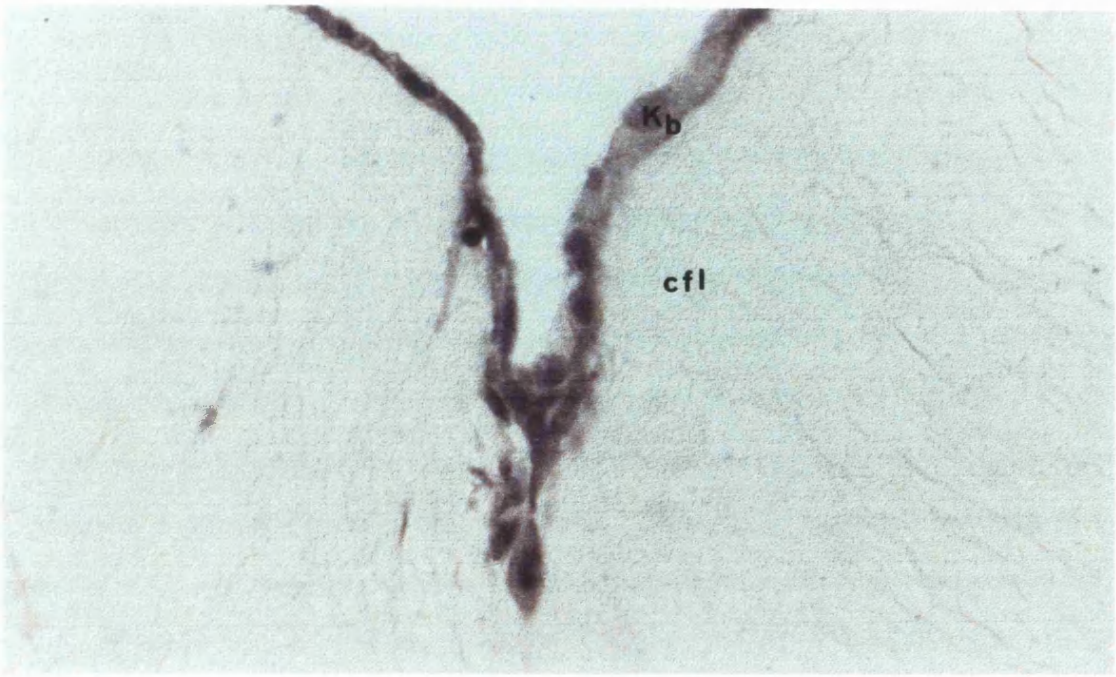


Figure 2.6c *Day 21*

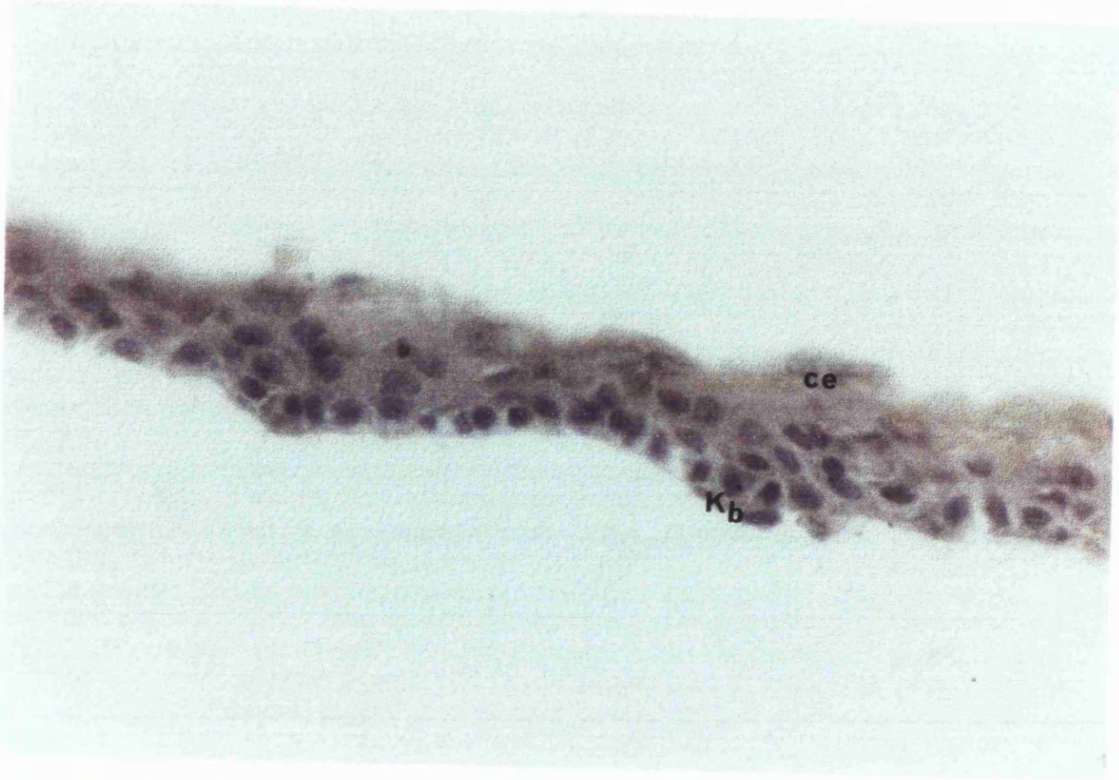


Figure 2.6d *Day 28*

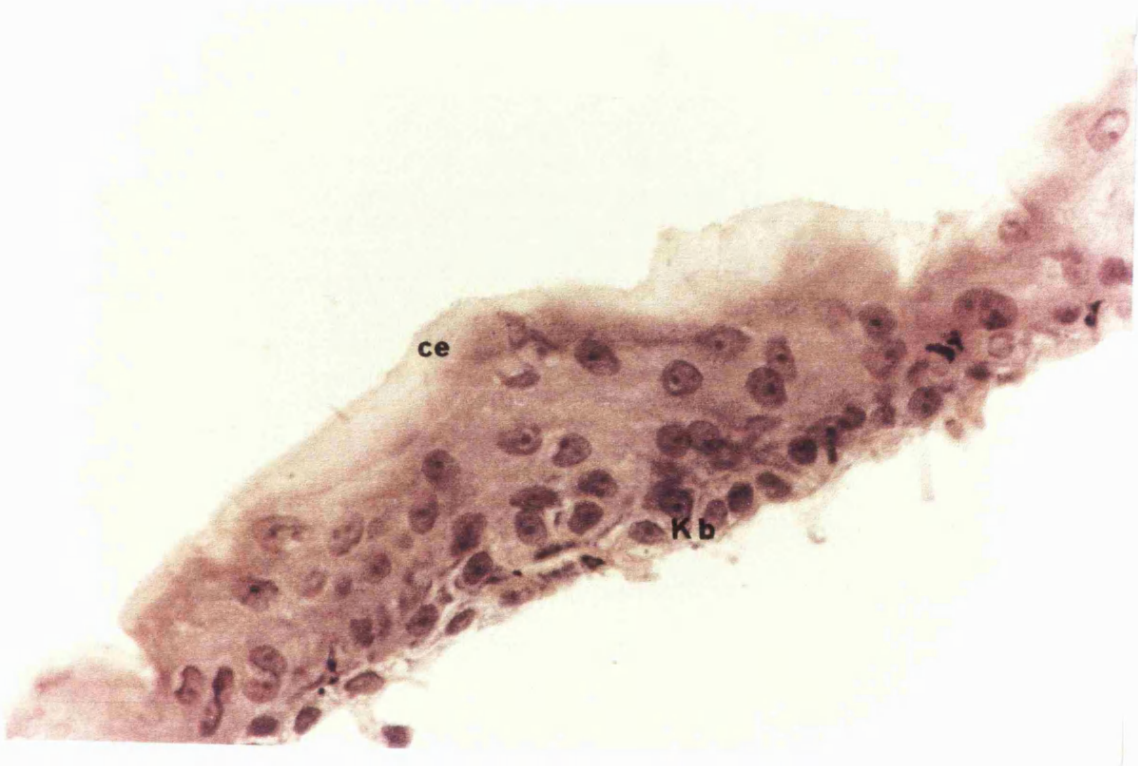
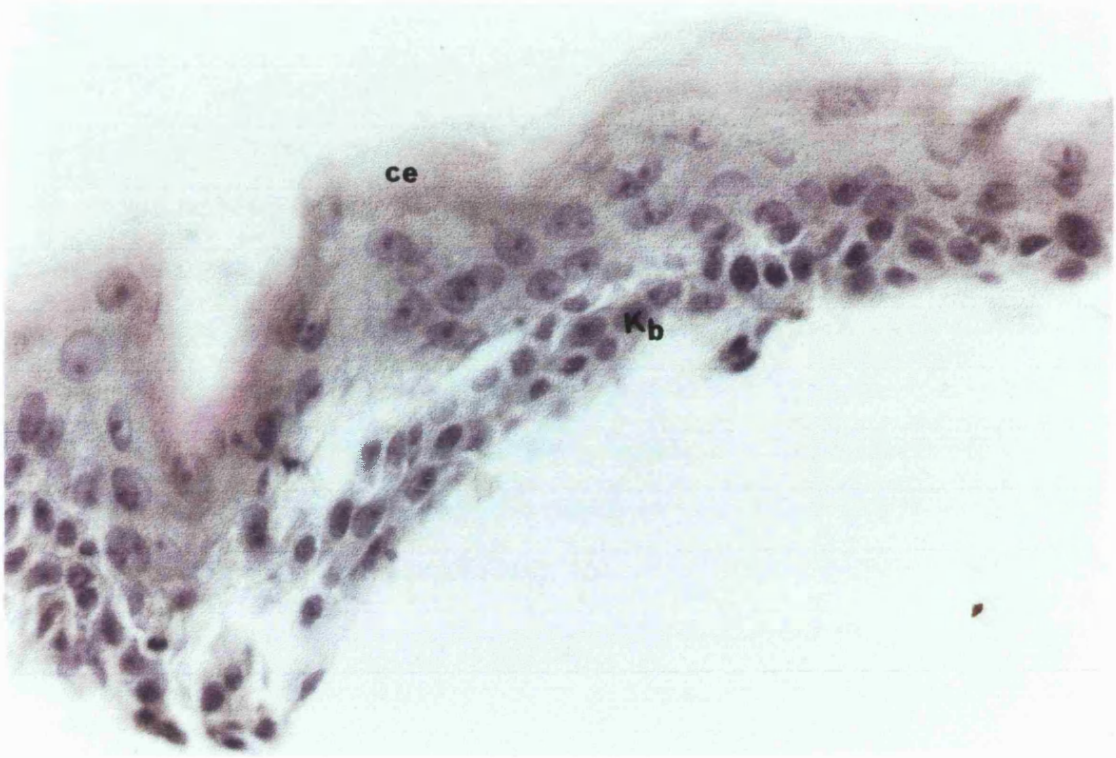


Figure 2.6e *Day 42*



cfl: collagen fibroblast lattice; Kb basal keratinocytes; ce cornified envelopes

2.3.3 Keratin production and location

Placing the SE at an air-liquid interface induces differentiation and stratification as shown by the presence of keratins K1 and K10. These keratins were absent in the basal layer of normal skin (figure 2.7a) and a faint fluorescence was detected in the granular and spinous cell layers whilst a strong intense stain was seen in the cornified layer of normal skin.

In the SE (figure 2.7b) although the basal cell layer did not appear to produce any K10, the cornified layer produced an even and intense fluorescence however, there was not much in the way of faint fluorescence suggesting either an absence or deficiency in the granular and spinous cell layers or a delayed production of K1/K10. Identification and correct location of involucrin and filaggrin may give an indication of the presence and size of the spinous and granular cell layers respectively.

Keratins extracted from skin, NHK and SE were separated using the technique of SDS-page and transferred electrophoretically on to nitrocellulose using Western blotting. These blots were incubated with AE3 which is an antibody for acidic keratins. The blots confirm the presence of K1 in the epidermis of skin and SE and highlight the reduced ability of NHK grown as submerged cultures to synthesise K1 and K10, reflecting a lower percentage of differentiated cells in this sample (see table 2.8). Keratin 5 was present in all samples whilst K6 was present in NHK in culture, and low levels were seen in keratinocytes grown on a DE reflecting the hyperproliferative nature of keratinocytes in these samples. The presence of K8 in skin samples has been attributed to the presence of

this keratin in the outer root sheath of hair follicles (Moll 1992). Thus since keratins are found in pairs it may be concluded that keratinocytes on the SE are able to synthesise K1, K2, K5, K10, K11 and K14 as seen in normal skin, but also a small amount of K6 and K16 which are associated with hyperproliferative skin.

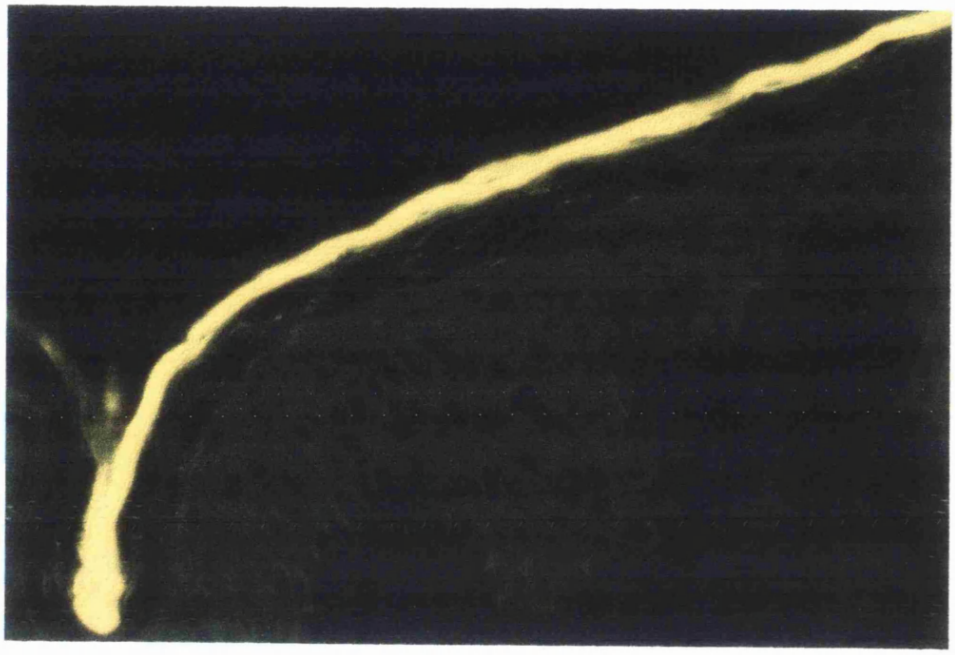
Table 2.8 *Keratins found in skin, and NHK in submerged and air-liquid cultures*

Sample	K1	K2	K5	K6	K8
Human skin	✓	✓	✓	✗	✓
NHK RM	✓↓	✓↓	✓	✓	✗
SE (NHK)	✓	✓	✓	✓↓	✗
SE (HFE)	✓	✓	✓	✓↓	✗
DE	✗	✗	✗	✗	✗

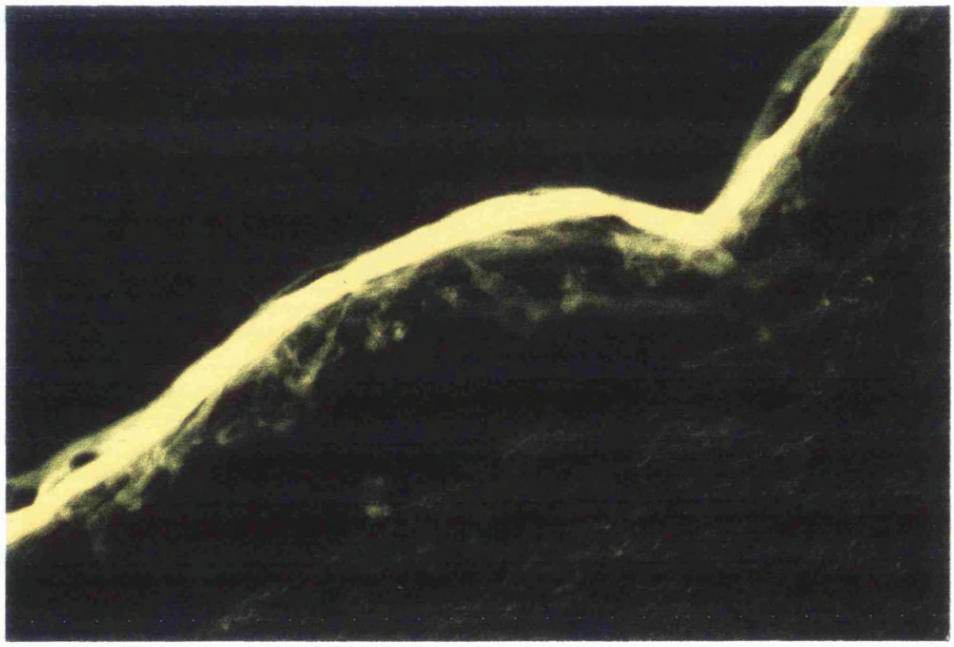
NHK RM= normal human keratinocytes derived from hair follicles grown in Rheinwalds medium SE =skin equivalent grown as described in methods (NHK seeded normal human keratinocytes, HFE implanted hair follicle explants) for 28d. DE= dermal equivalent grown as SE for 28d. See appendix IV for blot.

Figure 2.7 *Normal human skin (a) and Skin Equivalent (b) stained for K1*

(a)



(b)

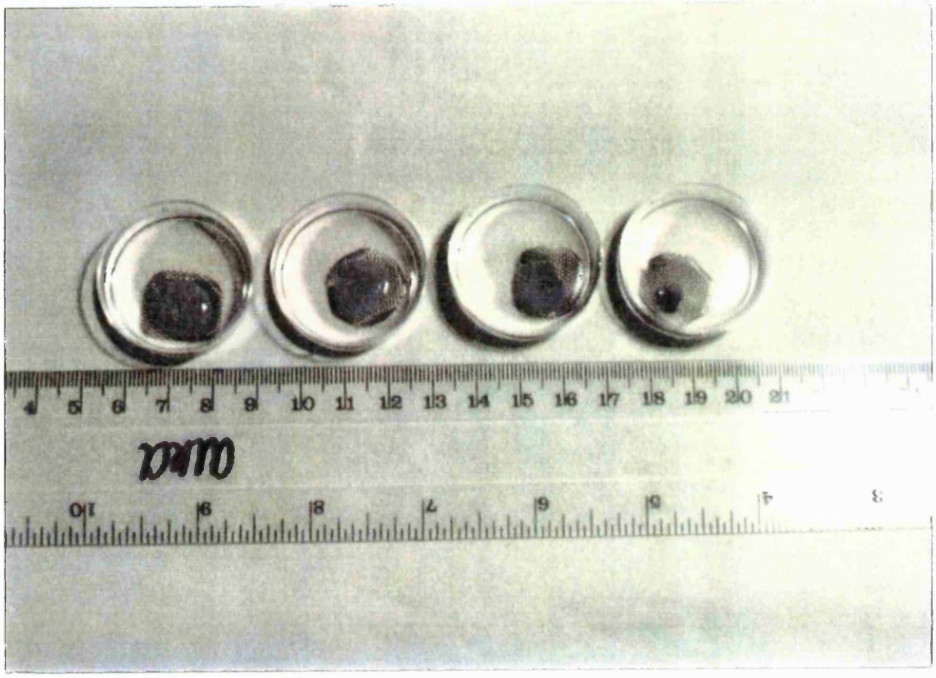


K1 is located by the presence of intense fluorescent yellow staining. Both the skin (a) and the skin equivalent contain high levels of K1 in the uppermost layers. However a gradual increase in the fluorescence is seen in the skin sample but is absent from the skin equivalent indicating the absence of granular and spinous layers in the synthesis sample.

2.3.4 Effect of calcium on the growth of keratinocytes on a DE grown in serum-free medium

As yet the growth of a SE comprising a collagen-fibroblast lattice in serum-free medium does not appear to have been attempted. In the same way that in some cases it is highly advantageous to eliminate indefinable serum constituents in keratinocyte monolayers, it may also be interesting to know whether or not keratinocytes continue to grow on a DE in a defined serum-free medium. The effect of increasing concentrations of calcium on epidermal growth was assessed by staining with nile blue sulphate. These studies show it is possible to grow keratinocytes from hair follicle explants on a collagen-fibroblast DE in serum-free medium (KSFM). Epithelial growth was monitored on the DE by staining for phospholipid in the cell membrane of keratinocytes on the DE. The effect of calcium was monitored by growing the SE in KSFM in the presence of increasing concentrations (0.9 mM - 1.4 mM) of calcium. Figure 2.8 shows an increase in the intensity of the blue stain with increasing concentration of calcium. Thus like keratinocytes grown in submerged cultures, keratinocytes cultivated as organotypic culture are also able to grow in serum-free medium. The increase in nile blue staining indicates an increase in the amount of phospholipid present suggesting the presence of more cells with increasing calcium concentration. The state of maturation of these cells has yet to be investigated.

Fig 2.8 *Effect of calcium on SE grown in serum free medium*



Skin equivalents grown in serum free medium (KSFM) with increasing concentrations of calcium: a = 0.09 mM, b =0.5 mM, c =1.0 mM and d = 1.4 mM. Skin equivalents stained with Nile blue sulphate are shown in petri dishes, on stainless steel grids. As the level of calcium is raised there is an increase in the intensity of the blue stain indicating an increase in epidermal thickness most likely to due to an increase in cornified layers as calcium has been shown to increase cellular differentiation at high concentrations (see text)

2.4 CONCLUSIONS

SE derived from hair follicle explants gave rise to an irregular epithelial layer. This was attributed to differences between individual outer root sheath of hair follicles synthesising different numbers of NHK per SE. Therefore the system was developed so that a quantified number of NHK, always at passage 3 were seeded onto a DE. The resulting SE produced a more regular epithelium, and this was reproducible. The NHK were derived from the outer root sheath of hair follicles rather than fresh skin. All materials used to synthesise the SE were readily available and this makes the system accessible to any laboratory with normal tissue culture facilities. The SE is more representative of the epidermis than cultures immersed in monolayer since:

a) a multi-layered epithelium was present representing the whole epithelium rather than the basal cell layer alone,

b) keratinocytes were able to differentiate at the air-liquid interface as assessed by the presence of a cornified layer and keratins 1 and 10,

c) all keratins present in normal skin are present K1, K2, K5, K10, K11 and K14.

However histology studies indicated that the SE is rather hyperproliferative since nuclei could sometimes be seen in upper cell layers of the epithelium and this was confirmed by the presence of K6. The attachment of the epithelial layer to the DE appeared poor relative to normal skin samples and this may be due to a poorly formed basement membrane. The basement membrane contains two types of collagen, type III adjacent to the matrix and type IV directly below the epithelial layer (Wheater 1987). Application

of a thin layer of one or both of these types of collagen on the upperside of a contracted DE prior to seeding keratinocytes may improve adhesion between dermal and epidermal layers. Poor connection may also be due to low levels of fibronectin, integrin $\alpha_6\beta_4$ or bullous pemphigoid antigen which help connect basal cells to the basal lamina (Carter 1990). This could be investigated using electron microscopy techniques.

The presence and correct location of various differentiation markers such as involucrin, loricrin, filaggrin and epidermal transglutaminase have as yet not been carried out. Since the model of normal skin has been set up so that it may be adapted to produce a model of initiated skin, further studies characterising the SE need to be carried out with normal and the initiated SE simultaneously.

CHAPTER THREE

Development of an assay for tumour promoters: I. The source of initiated human keratinocytes

3.1 INTRODUCTION

According to Hennings a valid model of initiated epidermis requires that:

a) the clonal expansion of initiated cells is suppressed by the surrounding normal cells and,

b) that this inhibition should be overcome on exposure to tumour promoters allowing the clonal expansion of initiated cells (see figure 1.8).

These properties were exhibited by an *in vitro* analogue of initiated mouse epidermis developed by Hennings and co-workers in 1990, in which a small number of initiated cells (308), were cultured in a confluent monolayer of normal mouse keratinocytes. Cell line 308 was derived from calcium resistant foci of keratinocytes from adult Balb/c mouse skin which had been initiated *in vivo* with DMBA.

A variety of phorbol ester-type (TPA, mezerein, teleocidin and aplysiatoxin) and non-phorbol ester-type (benzoyl peroxide, okadaic acid and staurosporin) tumour promoters all induced the clonal expansion of the purportedly initiated 308 keratinocytes (identified by staining with crystal violet or rhodamine) when co-cultured amongst a confluent monolayer of normal mouse keratinocytes (Hennings et al 1992). Furthermore the anti-promoters retinoic acid (RA) and fluocinolone acetonide (FA) reduced the number of 308 colonies when added concurrently with TPA (Hennings et al 1990).

308 growth suppression by normal keratinocytes was reported to require cell to cell contact since medium conditioned by primary confluent keratinocytes was unable to suppress 308 growth. Further, neither the number, or the size of 308 colonies was

affected by co-culture with normal fibroblasts in place of normal keratinocytes, indicating that inhibition of 308 cell growth was cell type specific.

The inhibition of 308 colonies only occurred when co-cultures were grown in medium containing a high concentration of calcium (1.2 mM) and not in medium containing low concentration of calcium (0.05 mM) (Hennings 1990, 1991). Thus this system requires the presence of high levels of calcium and is therefore different from GJIC systems in which maximum inhibition occurs at low concentration (see chapter 1 section 1.5.1.2).

The mechanism involved in this model therefore, appears to be calcium dependant and requires cell to cell contact but does not appear to directly involve junctional communication. Dose response curves for 308 cells in co-cultures exposed to tumour promoters differed from dose response curves of 308 cells cultured alone with the same promoters suggesting the promoter activity occurs via the normal keratinocytes.

Although this system still requires extensive validation, preliminary studies look promising. The model described appears to be a good representation of initiated mouse skin *in vivo* with respect to the identification of tumour promoters and anti-promoters. However this system has limited advantages in predicting tumour promotion in humans. Not only is there a need to compensate for species differences but this co-culture also requires *in vitro* to *in vivo* extrapolation making the model less desirable scientifically than mouse *in vivo* systems. Since the *in vivo* rodent data appears to closely match the *in vitro* rodent data it would be plausible to believe that a similar co-culture of normal and

initiated human keratinocytes *in vitro* may give rise to a more accurate indication of tumour promotion in human skin *in vivo* than rodent models.

3.1.1 A source of initiated human keratinocytes

In order to set up a model of initiated human epidermis there was a need to find a source of normal and initiated human keratinocytes. As indicated in chapter 2, NHK may be derived from human skin explants or from the outer root sheath of hair follicles. Due to the irregular supply of skin, normal keratinocytes were derived from hair follicles using a variation of the method described by Limat and Noser (1986, and see methods).

Initiated rodent keratinocytes may be obtained either by exposing rodents to initiators *in vivo* and then selectively culturing initiated cells, or by exposing normal rodent keratinocytes to initiators *in vitro*. Rodents are more prone to cancers than humans, possibly due to the high stability of the human genome (Rhim 1989). This may also explain why human cells are so difficult to initiate *in vitro.* In setting up a co-culture of human initiated epidermis to be used as an assay, it was essential that only readily available sources were used and also, that the assay was simple to carry out. Therefore initiated human cells were substituted for a cell line which displayed the pre-malignant phenotype in the model of initiated human epidermis. This chapter describes the characterisation of three cell lines assessed for their suitability in representing initiated cells. The criteria for which includes:

a) that the cells must be non-malignant

b) they must be immortal and

c) that they do not, or only partially differentiate, in response to normal cell differentiation stimuli.

3.1.2 Characteristics of the initiated cell phenotype

Three cell lines, HK-2, HK-3 and HK-4 human keratinocytes which had been immortalised by transfection with a mutant subgenomic SV40 gene using an amphotropic retroviral vector (kindly donated by M O'Hare), were investigated for inclusion as initiated cells in the co-culture assay for human skin tumour promoters.

3.1.2.1 Morphology

Initiated cells are morphologically similar to normal cells and thus the prospective cell line should resemble basal keratinocytes as this is the sub-type of keratinocyte that is initiated in basal and squamous cell carcinoma.

3.1.2.2 Premalignant phenotype

An essential feature is the need for the chosen cell line to display a premalignant phenotype. Cells can be distinguished as being normal, initiated (premalignant) or cancerous (malignant) following inoculation into athymic mice. Normal cells will grow to give rise to a normal epithelium whilst carcinogenic cells characteristically induce malignant tumours and initiated cells may produce benign papillomas.

3.1.2.3 Proliferation

In skin, papillomas are characterised by a high rate of proliferation. The chosen cell line should have a faster growth rate *in vitro* than normal cells, analogous to initiated cells *in vivo*.

3.1.2.4 Ability to differentiate

The SV40 gene is used to extend the normal life span of cultured human cells, and thereby generate cell lines. One major disadvantage is that often normal differentiative processes are suppressed and the resulting cell line no longer functions as the parent cell. In this case all cell lines were transfected with a sub genomic SV40 gene in an attempt to reduce the loss in differentiation capability. The cell line chosen should possess the ability to differentiate. This may be monitored by the production of keratins 1 and 10, which are known as markers of differentiation in keratinocytes, using the technique of SDS-page.

3.1.2.5. Reluctancy to differentiate following exposure to inducers of differentiation

Although capable of differentiating, the initiated cells characteristically show a reluctancy to differentiate following exposure to inducers of differentiation such as TPA (see chapter 1, section 1.3.2.2).

(i) Cultivation at the air-liquid interface

Normal cells are induced to differentiate upon seeding onto a collagen lattice at the air-liquid interface (see chapter 2). All cell lines were cultured on a DE at the air-liquid

interface and their ability to continue to synthesise K1 and K10 on the DE was investigated and compared with K1 and K10 production by the same cells grown in monolayer using the techniques of SDS-page and immunofluorescence.

(ii) TPA induced differentiation

TPA induces differentiation of 90-93% of normal cells (see section 1.3.2.2). Initiated cells show resistance to the induction of differentiation. Thus the chosen cell line should show a reduced sensitivity to TPA induced differentiation. The capacity of each cell line to differentiate was assessed in two ways, (a) by comparing the colony forming efficiency and (b) cornified envelope formation of the cells in the presence and absence of TPA.

3.1.2.6 Effect of TPA on a co-culture of NHK and HK cells

In order to conclusively test the suitability of each of the cell lines for incorporation into the human model of initiated epidermis co-cultures of NHK and each of the three HK cell lines were grown in the presence of TPA for 14 days.

3.2. METHODS

Materials see Appendix (i)

Media see Appendix (ii)

Culture of keratinocytes derived from the outer root sheath see chapter 2.

Apart from cornified envelope experiment (n=1), all data and statistics may be found in appendix

3.2.1 Growth rates

Approximately 1 x 10^5 cells were seeded into each of 16 (35 mm diameter) culture dishes and in RM+ medium which was changed 3-4 times/week. Two wells were trypsinised and cells counted using a haemocytometer at days 1,2, 3, 6, 7 and 8.

3.2.2 Colony forming efficiency

HK cells were plated into 6 well plates either with (500 HK cells) or without (2500 HK cells) a 3T3 feeder layer and cultured in the presence or absence of 1 nM or 20 nM TPA. The number of colonies larger than 1 mm were counted after fixing in formalsaline and staining for 2 minutes in haematoxylin. Each experiment was repeated 3-5 times.

3.2.3 Cornified envelope formation

Cornified envelope formation was measured according to the method of Parkinson 1983.

3.2.4 Co-cultures of NHK and HK cells

100 HK cells were added to a culture dish (60 mm diameter) with 1 x 10^5 NHK in Rheinwalds medium (RM+C see appendix II) in the presence of choleratoxin. 10 nM TPA was added to these co-cultures 24 hours after plating and continued for 14 days.

3.3 RESULTS AND DISCUSSION

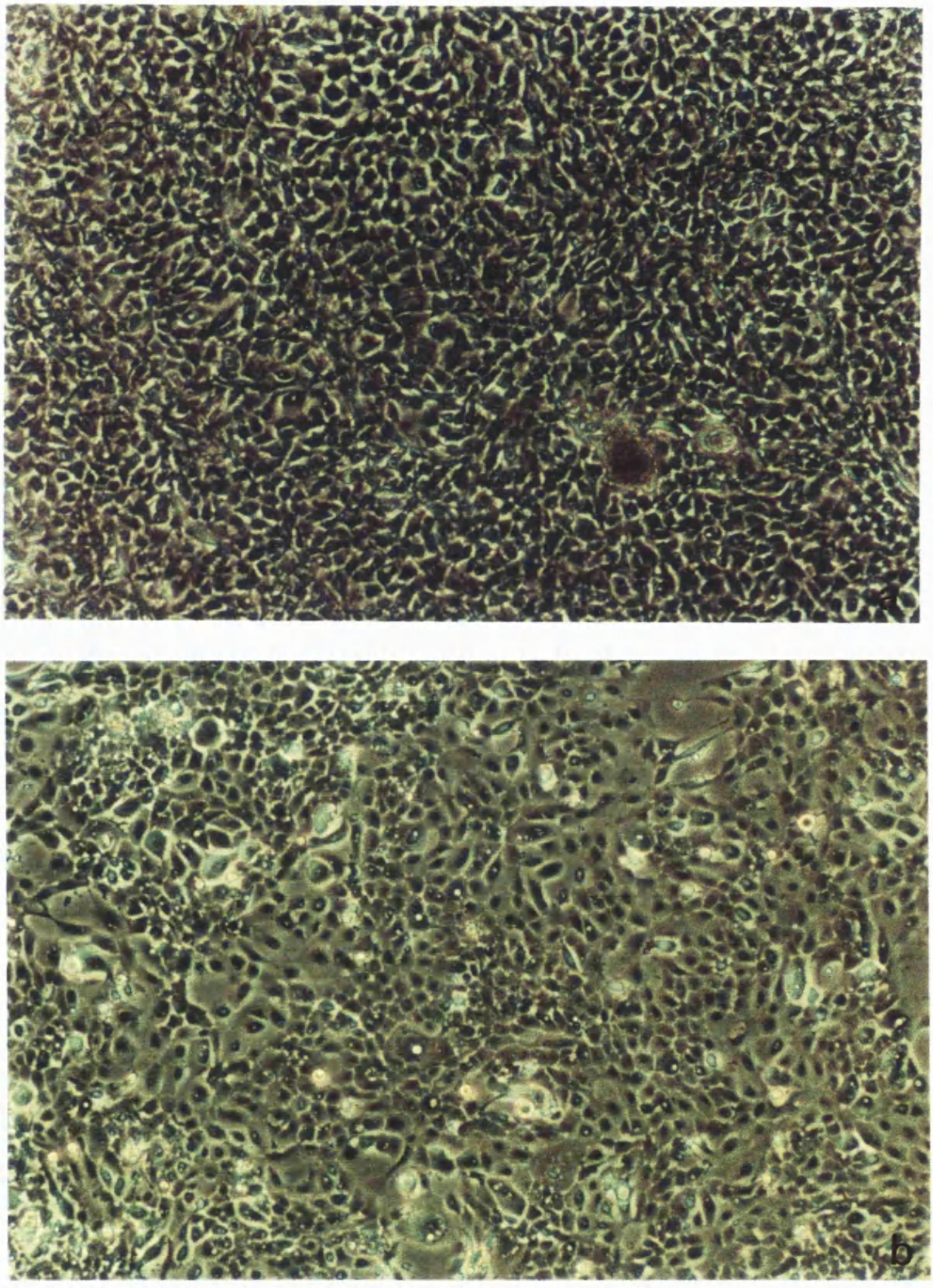
3.3.1 Morphology

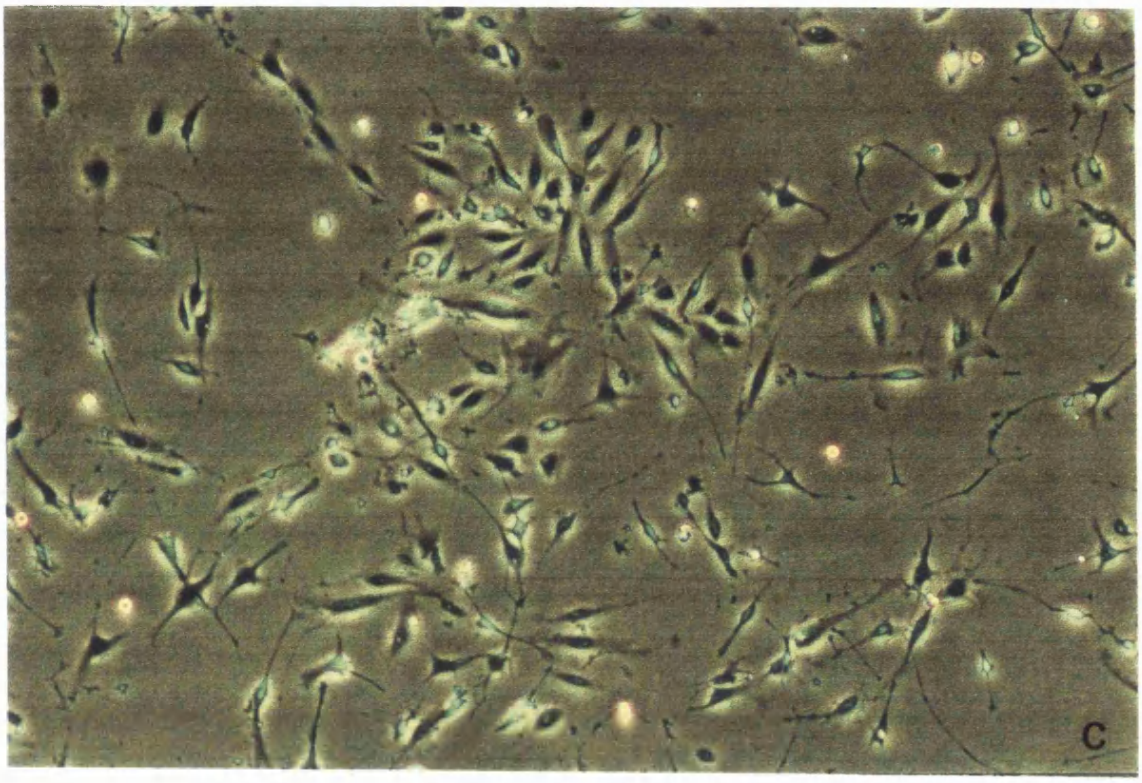
Normal keratinocytes were grown in serum-containing keratinocyte growth medium, whilst all three keratinocyte cell lines were cultured in the same medium with the omission of choleratoxin which retards their growth. Of the three cell lines, HK-2 (figure 3.1(b)) and HK-4 (figure (3.1 d)) cells resembled normal keratinocytes (figure 3.1(a)), displaying the characteristic cobblestone appearance of basal cells when grown in submerged culture. Both cell lines HK-2 and HK-4 were slightly smaller than NHK and HK-4 cells were more homologous in their appearance than HK-2 cells. HK-3 keratinocytes showed little resemblance to NHK. These cells showed morphological similarity to fibroblasts in that they were elongated and spindly in their appearance (figure 3.1(c)).

3.3.2 Premalignant phenotype

All three cell lines produced small 2 mm nodules following inoculation in nude mice. These nodules subsequently regressed indicating the cell lines all displayed the premalignant phenotype (personal communication M O' Hare).

Figure 3.1 *Morphology of NHK and HK cell lines under phase contrast microscope. a) NHK, b) HK-2, c) HK-3 and d) HK-4 (see 3.3.1).*

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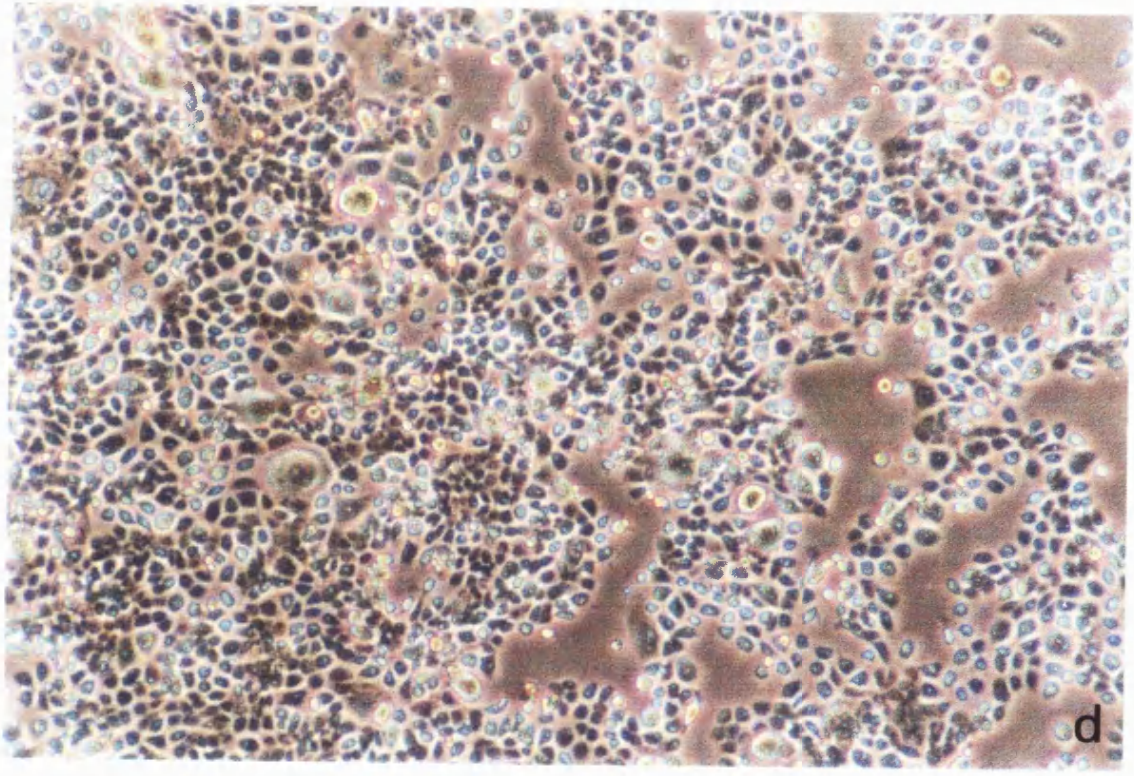
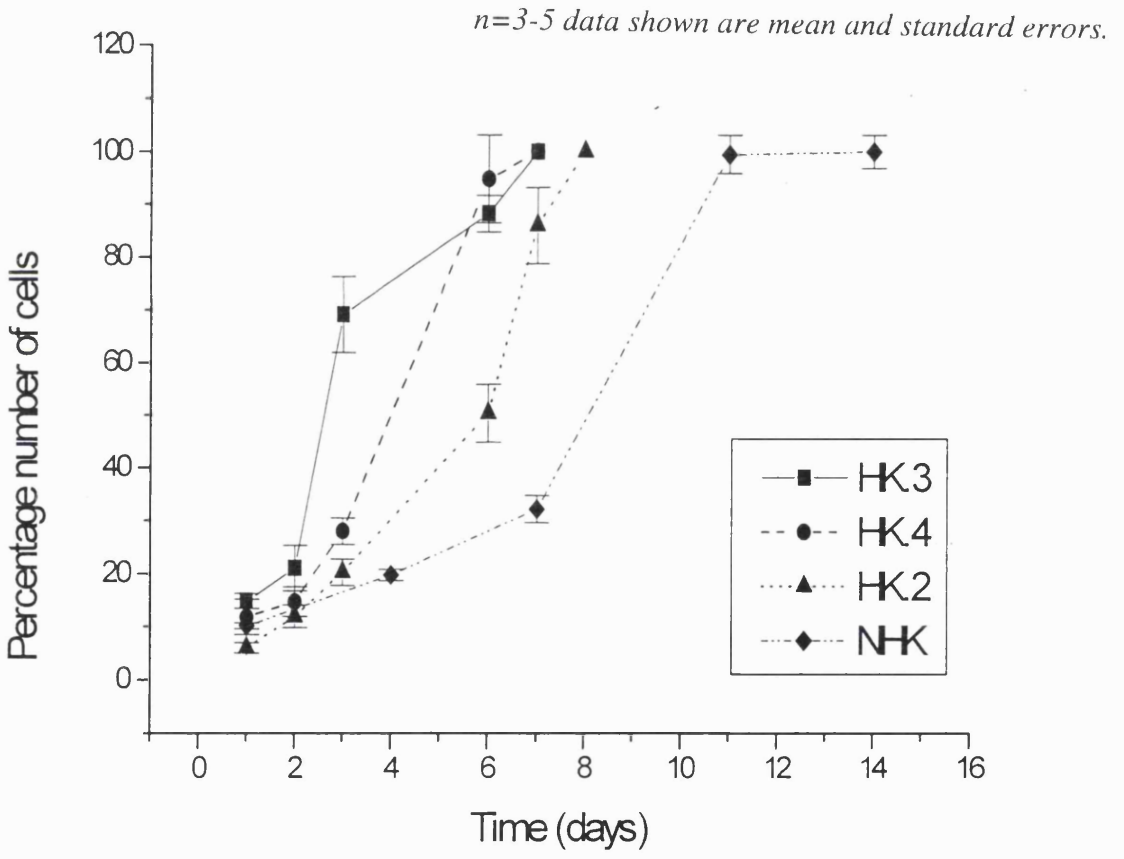

d

3.3.3 Proliferation

Growth rate studies indicated all three cell lines have a faster growth rate than NHK. This is mainly due to a long lag phase seen with normal but not transformed cells.

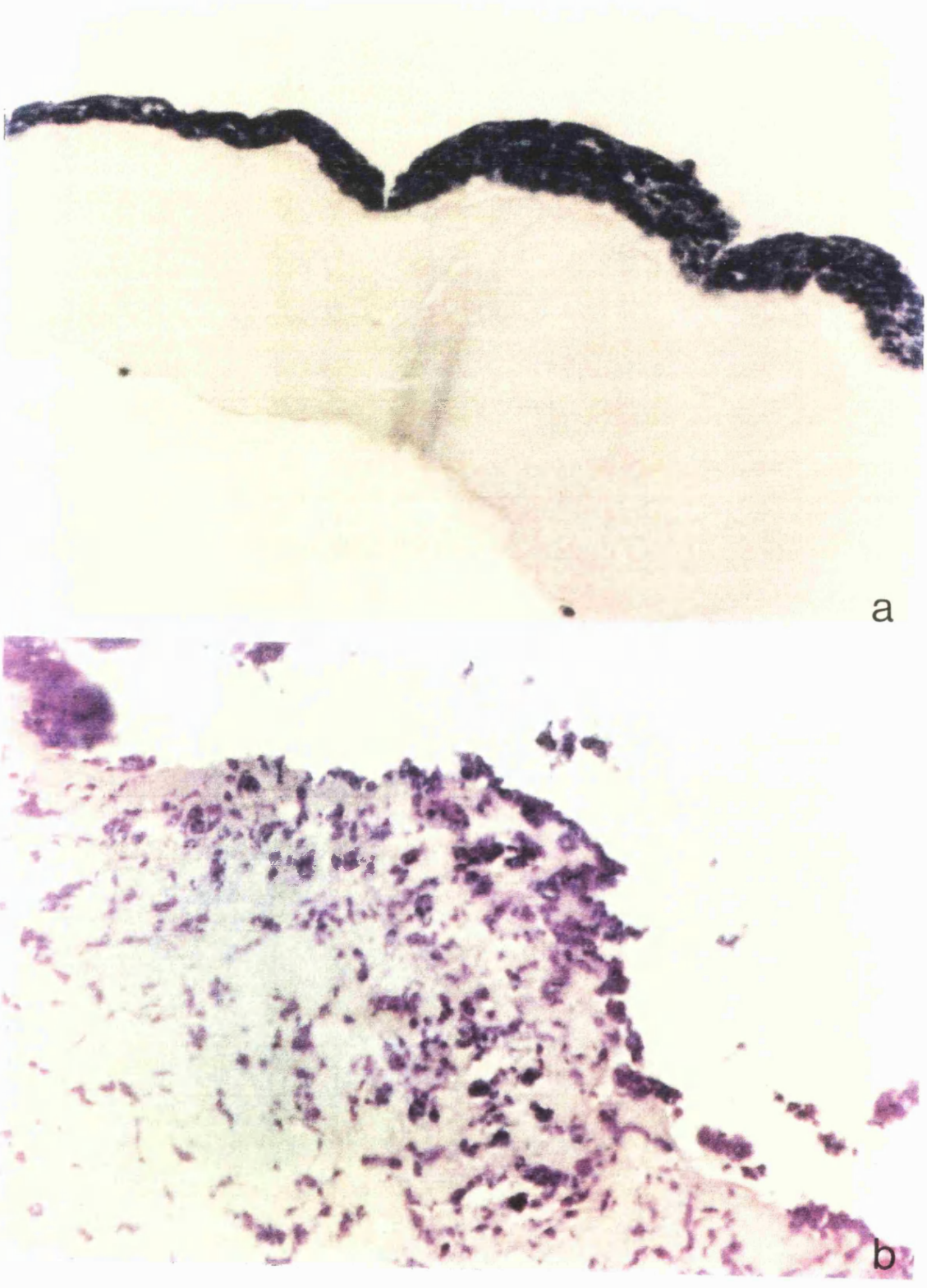
Figure 3.2 *Growth rates of NHK and HK cell lines*

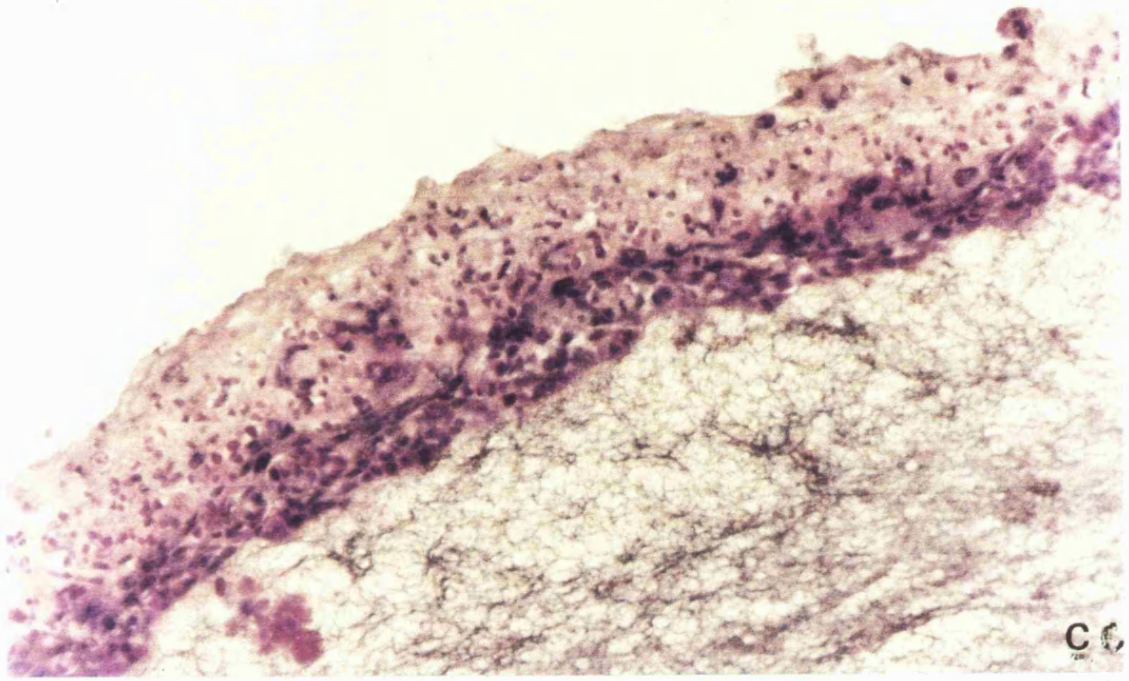


3.3.4 Ability to differentiate

When grown on a dermal equivalent, HK-4 cells produced a continuous multi-layered culture of 10-15 cells in depth (figure 3.3 c) The culture appeared hyperproliferative compared with normal keratinocytes since nucleated cells could be seen in the upper epidermal layers and the cornified layer was poorly formed. Similarly HK-2 cells also produced a continuous multi-layered epidermis (figure 3.3a), which was only 2-5 cell layers thick. A cornified layer was however not present. HK-3 cells produced a rather erratic multi-layer (figure 3.3b) which penetrated the dermal substrate and showed little resemblance to hyperproliferative skin. These cells appear to have a fibroblastic nature as well as morphology.

Figure 3.3 *NHK and HK cell lines grown at the air-liquid interface (day 28) (a) HK-2, (b) HK-3 and (c) HK-4.*





H&E stained vertical sections of skin equivalents, DE= dermal equivalent, EP = epithelial layer.
a) *Individual HK-2 cells can be identified by intensely stained blue nuclei. These cells form a stratified epithelial layer on the dermal equivalent. There appears to be no loss of nuclei in uppermost layers suggesting poor differentiation.*
b) HK-3 cells have penetrated the dermal compartment and unlike HK-2 and HK-4 cells HK-3 cells do not produce stratified epithelial layers.
c) Like HK-2 cells nucleated HK-4 cells can be seen attached to the dermal equivalent forming epithelial cell layers above the collagen-fibroblast dermal compartment. The uppermost layer of keratinocytes appears less basophilic and more eosinophilic suggesting that the outer layers have lost their nuclei indicative of keratinocyte differentiation (also see 3.3.4).

3.3.5 Keratin pattern

(a)Submerged culture

SDS-page studies of keratinocytes in submerged culture demonstrated that all HK cell lines, like normal keratinocytes, expressed the basic keratins K1, K5 and K6 in culture (Table 3.1). However expression of K1 was reduced and, in addition, all HK cell lines strongly expressed K8 which is representative of simple epithelial cells and indicates that the cells have proceeded towards a malignant phenotype (Moll et àl 1982, Smack et al 1994). No K8 was detected in normal cultured keratinocytes although previous reports have recorded expression of this keratin in 0.1-1% of human keratinocytes (Darmon et al 1984). This production of K8 by NHK has been linked to stem cells. Since the keratinocytes used in the electrophoresis and immunohistochemical studies (and also those used for co-culture studies) had been subcultured through three passages in a high calcium medium which favours cellular differentiation the number of stem cells would be far reduced in these keratinocytes and therefore one may expect reduced levels of K8 in cultured keratinoytes compared with keratinocytes *in situ.*

(b) Air-liquid interface culture

Upon selectively culturing cells at an air-liquid interface, all cell lines demonstrated an increased synthesis of K1 (marker of differentiation) as shown by SDS-page staining for basic keratins. This was indirectly confirmed by immunofluorescence studies staining specifically for K10 (the acid keratin of the K1/K10 pair) (figure 3.4). Whilst all three cell lines had strongly expressed K8 in submerged cultures the synthesis of this keratin appeared to be reduced or eliminated when cells were cultured on a DE at the air- liquid

interface.

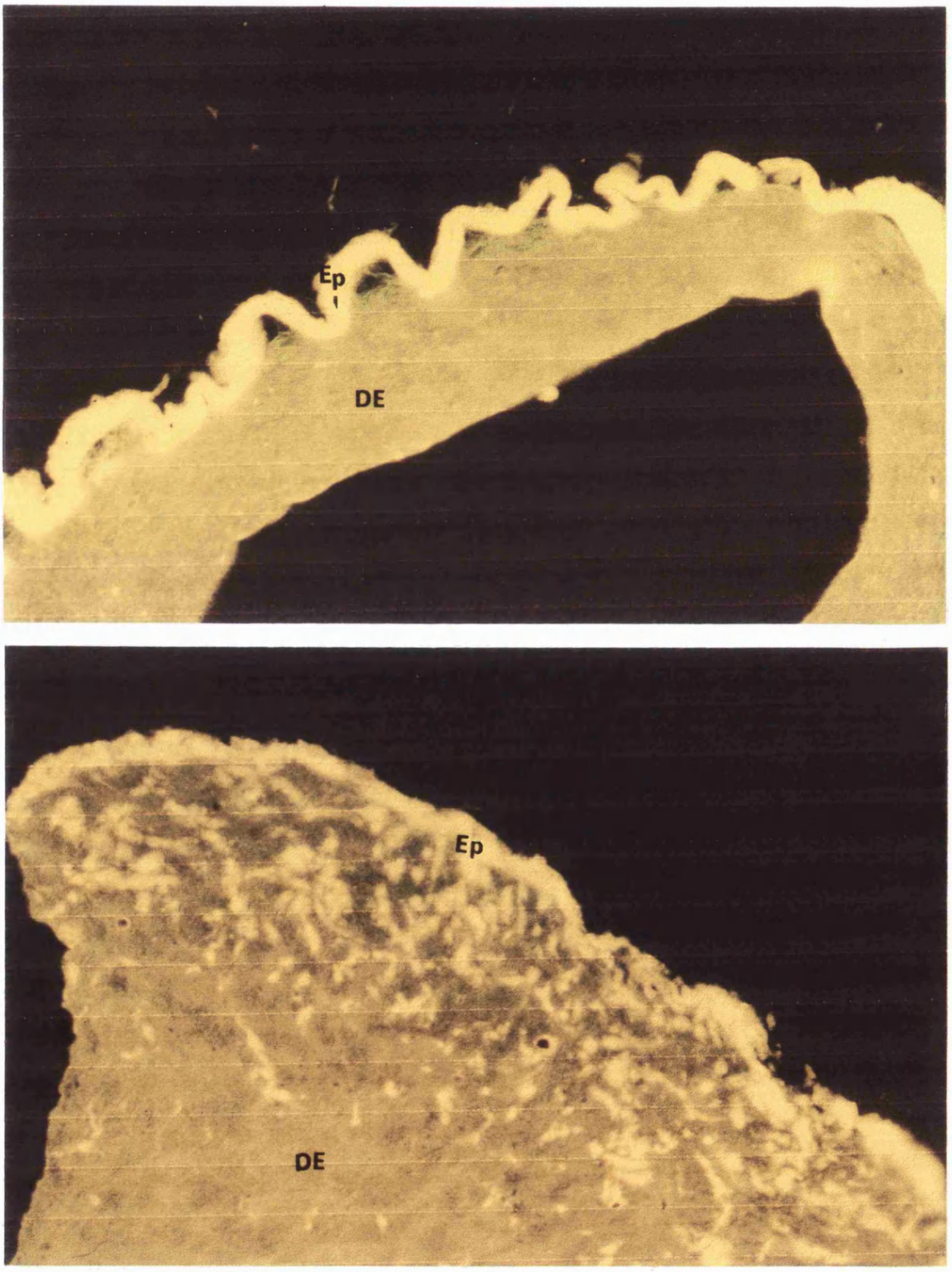
These results indicate that HK-2, HK-3 and HK-4 cells in submerged culture produce all the keratins synthesised by NHK in monolayer. In addition K8 which is representative of simple epithelia, is also present (the absence of K8 from NHK has been discussed 3.3.5.a). At the air-liquid interface, all keratinocyte cell lines were induced to differentiate as indicated by an increase in the K1/K10 keratin pair. They remain hyperproliferative as demonstrated by positive labelling with an antibody for K6. They also appear to be moving away from a simple epithelial phenotype as demonstrated by the absence of K8 in electrophoresis studies.

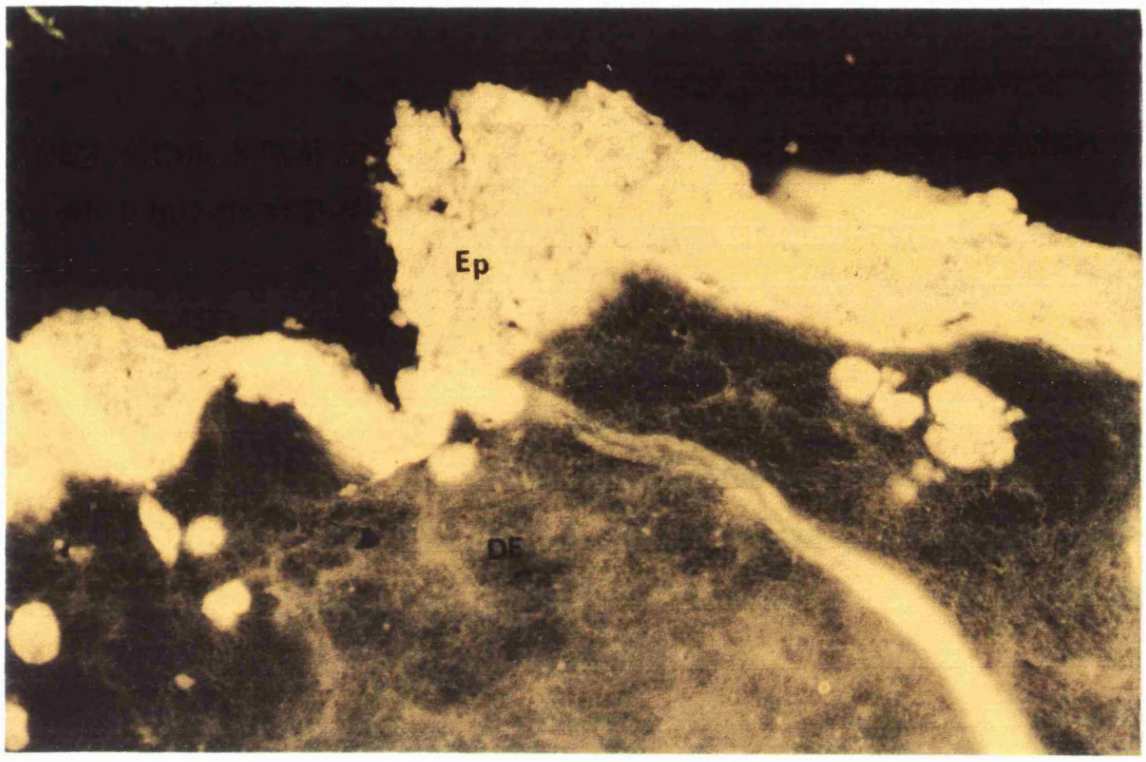
Table 3.1 *Keratin distribution in skin and submerged and air liquid interface cultures of NHK and HK cells*

Keratin	Human Skin	NHK in submerged culture	NHK at the air-liquid interface	HK cells in submerged culture	HK cells at the air-liquid interface
K1	✓	✓↓	✓	✗✗	✓
K2	✓	✓↓	✓	✓	✓
K5	✓	✓	✓	✓	✓
K6	✗	✓	✓(↓)**	✓	✓
K8	✓*	✗	✗	✓	✗

*The presence of K8 in human skin is likely to be derived from hair follicles (Moll 1982).
** Levels of K6 in the SE was reduced compared with other cultures.
See Appendix IV for blot.

Figure 3.4 *HK-cell lines grown on a dermal equivalent stained for K10 (a) HK-2, (b) HK-3 and (c) HK-4.*





DE=dermal equivalent, Ep=epithelial layer.Figures a-c are immunofluorescent HK-2, HK-3, and HK-4, SE stained for K10 (compare with H&E stains figure 3.3). A grey dermal equivalent can be identified by arrows, upon this sits a fluorescent layer made up of HK cells, indicating all these cells contain K10, the marker for keratinocyte differentiation.

3.3.6 Plating efficiencies

Like normal keratinocytes the plating efficiency of all three cell lines was enhanced in the presence of a 3T3 feeder layer (figure 3.5). To test whether the immortalised keratinocytes were more resistant to the effects of TPA than normal keratinocytes, the plating efficiency of each cell line was determined in the absence and presence of TPA (figure 3.6). TPA was added 24 hours after seeding the cells and the concentration of TPA was kept constant over a 14 day period.

The plating efficiency of HK-3 cells was unaffected by TPA at 5 nM and 20 nM. This is a further reflection of this particular transformed cell lines deviation from normal keratinocyte behaviour. Plating efficiencies of HK-2 and HK-4 were reduced to 41% and 47.8% of control values respectively. 90-93% of NHK have been reported to loose their colony forming ability when treated with 100 nM TPA for 24 hours (Parkinson et al 1983).

In our experiments when NHK were grown in 1 nM, 10 nM and 100 nM TPA, no colonies of NHK could be identified after 14 days continual exposure. Thus all three HK cell lines are more resistant than normal keratinocytes to the growth inhibitory effects of TPA. To ascertain whether this was due to a reduced responsiveness of these cell lines to the terminal differentiation stimulus of TPA, the extent of cornified envelope formation in these cell lines with and without TPA was determined.

Figure 3.5 *Plating efficiencies of HK cell lines with and without 3T3 feeder fibroblasts**

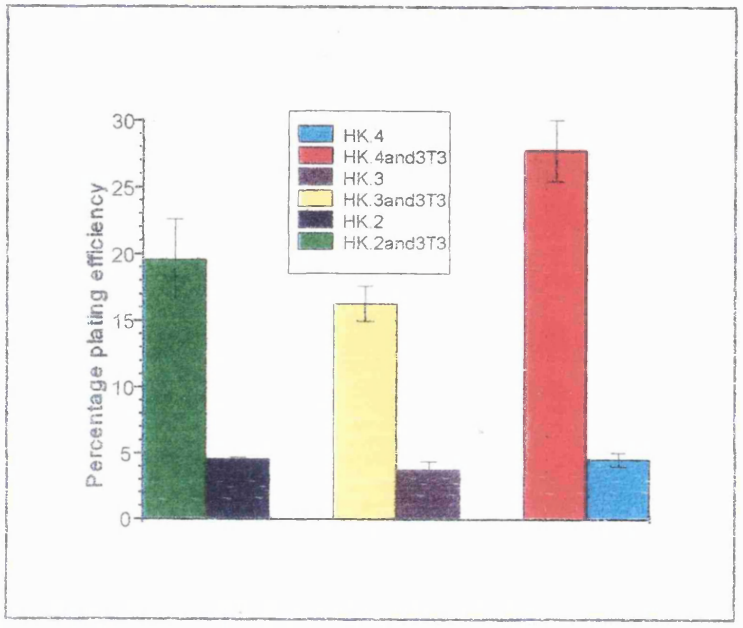
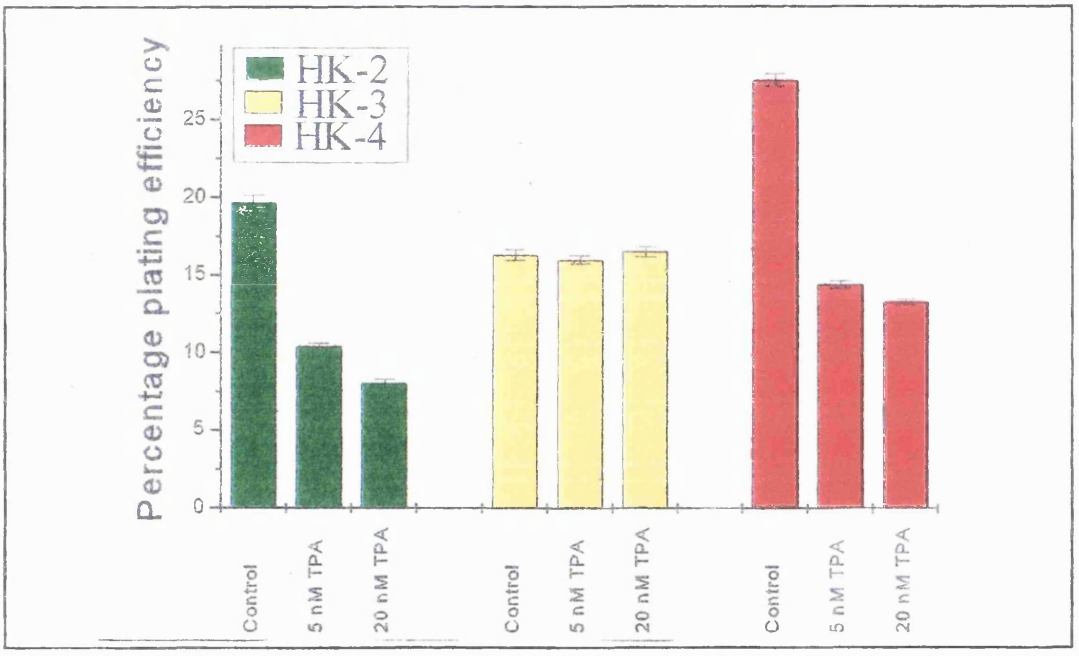


**Figure 3.6* Figure shows means and standard deviations of a minimum of 3 experiments, for data see appendix*

Figure 3.6 *Effect of TPA on the plating efficiency of the three human cell lines***



** *Figure shows means and standard errors of experiments conducted at least 3 times, for data see appendix*

3.3.7 Effect of TPA on cornified envelope formation

The cloning efficiency experiments indicated that the HK cell lines would have a growth advantage over NHK when grown in TPA containing medium. This is likely to be due to the promoter inducing differentiation of the normal cells. Parkinson (1983) showed that on exposure to TPA the number of NHK with cornified envelopes increased from 3% in control cultures, to around 70% in those treated for 6 days with 100 nM TPA. Whilst neoplastic and pre-neoplastic keratinocyte cell lines were less responsive to TPA producing only a 2-4 fold increase in envelope formation following TPA compared to their controls.

Between 0.5-1.1% of HK cells produced cornified envelopes after 2 weeks incubation in normal medium. Exposure of HK cell lines to TPA resulted in 3-5 fold increase in cornification by HK-4 and HK-3 cells, whilst HK-2 cells showed a drop in cornified envelope formation. These results suggest that all three HK cell lines should have a growth advantage over normal keratinocytes when exposed to TPA and that this growth advantage is likely to be due to a reduced responsiveness to the terminal differentiation stimulus of TPA.

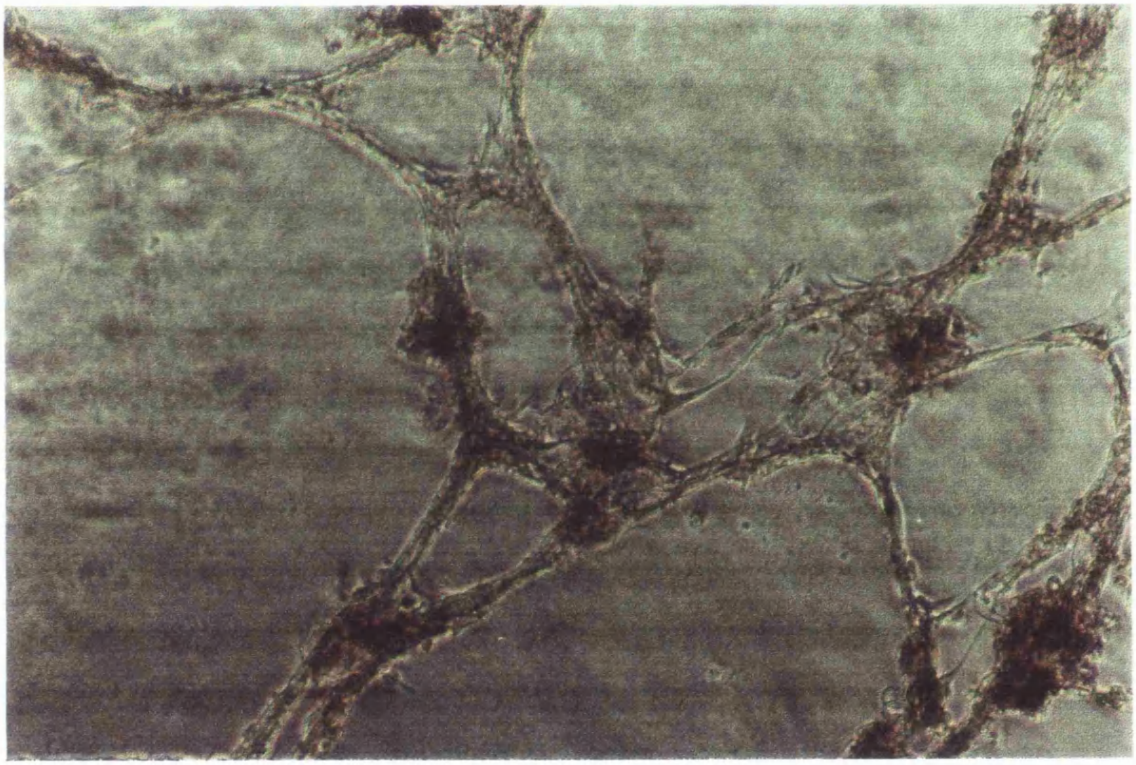
Table 3.2 *Percentage cornified envelope formation in the presence of TPA*

Cell line	Control	1 nM TPA	5 nM TPA	20 nM TPA
HK-2	1.1	1.6	0.8	0.9
HK-3	0.3	0.3	0.6	1.0
HK-4	0.5	2.3	2.6	1.8

3.3.8 Effect of TPA on co-cultures of NHK and HK cell lines

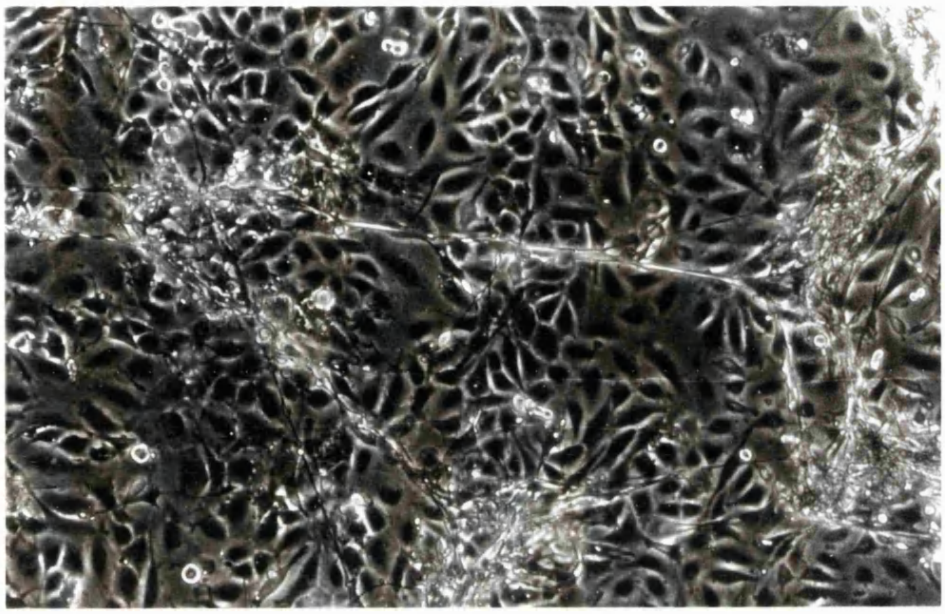
100 HK cells were cultured amongst a confluent monolayer of NHK for 14 days in medium containing 10 nM TPA. Both HK-2 and HK-4 cells formed tightly adherent small colonies as identified under a phase contrast microscope. HK-3 cells however did not form tight colonies but groups of individual HK-3 cells could be seen.

Figure 3.7a *Confluent NHK culture following 14 day exposure to 10 nM TPA*



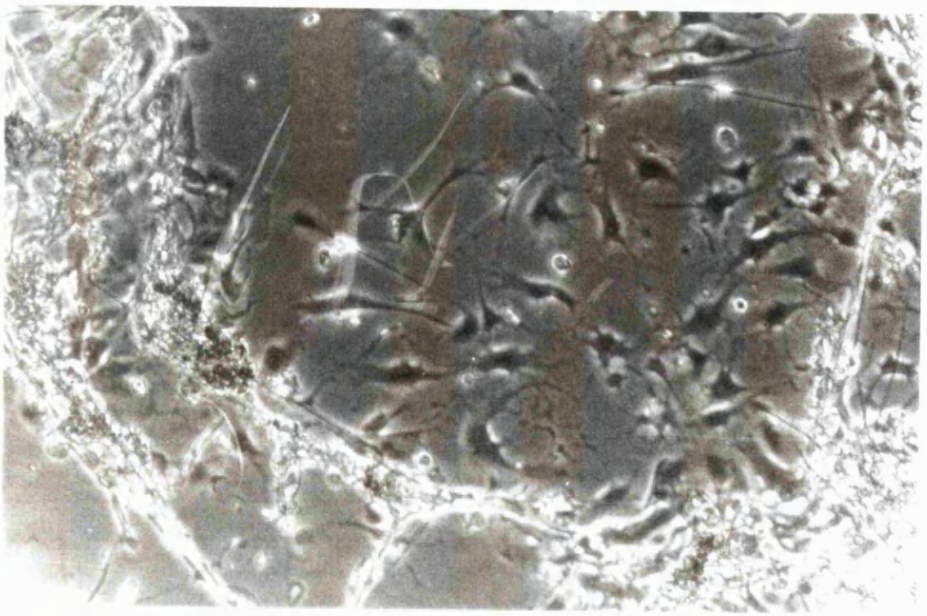
14 day exposure to TPA has induced differentiation of NHK (compared with normal NHK (figure 3.1a). Most cells have detached or have lifted away from the surface (analogous to cells moving away from the basal layer to supra basal layers)

Figure 3.7b *HK-2 and NHK co-culture following exposure to 10 nM TPA for 14 days*



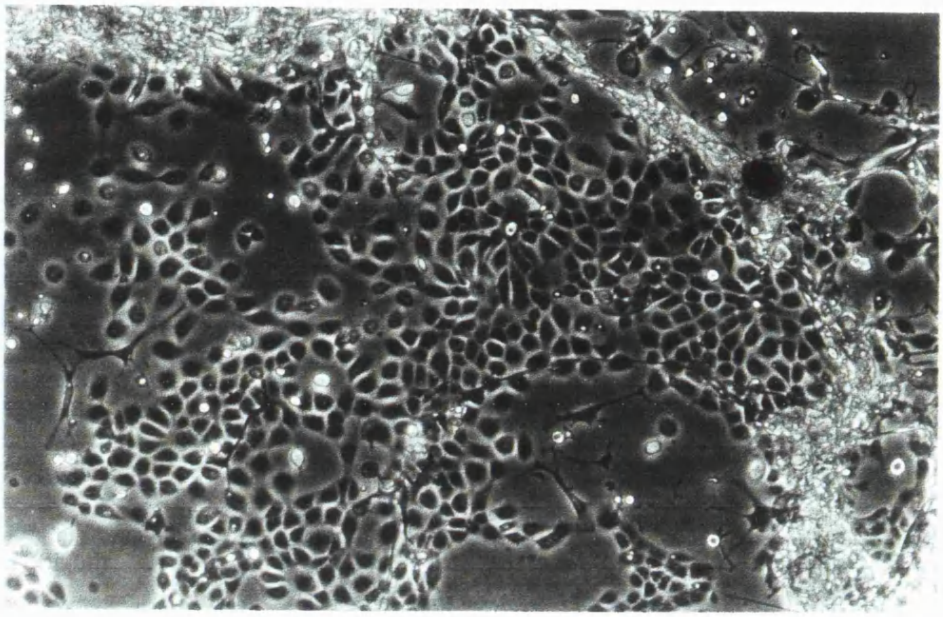
Differentiating NHK amongst proliferating HK-2 cells

Figure 3.7c *HK-3 and NHK co-culture following exposure to 10 nM TPA for 14 days*



HK-3 cells can be seen growing in spaces created by differentiated NHK. HK-3 cells, unlike HK-2 and HK-4 cells do not grow adjacent to one another but spread out in the space available in a similar manner to fibroblasts.

Figure 3.7d *HK-4 and NHK co-culture following exposure to 10 nM TPA for 14 days*



Colonies of HK-4 cells growing amongst the surrounding differentiated NHK

3.4 CONCLUSIONS

HK-2 and HK-4 cell lines showed similar morphology to NHK, growing in tightly formed adherent colonies, whilst the HK-3 cell line resembled fibroblasts spreading evenly rather than forming colonies, in submerged cultures. At the air-liquid interface HK-2 and HK-4 keratinocytes grew to form a stratified epithelium, however the resulting SE appeared rather hyperproliferative as a cornified layer was absent. HK-3 keratinocytes tended to penetrate the DE rather than forming an epidermal layer. The results from submerged and air-liquid interface cultures demonstrated the unsuitability of HK-3 cells. Firstly a co-culture model of initiated epidermis requires cell to cell contact, to be valid. Since HK-3 cells did not grow in colonies, cell to cell contact was reduced. Further, HK-3 cells did not form a representative epidermis when seeded at the air-liquid interface and therefore developing the submerged co-culture into an air-liquid culture could not be pursued using this cell line.

There was no difference in keratin patterns between the HK cell lines (HK-2, HK-3 and HK-4). However keratin production in these cell lines were different from normal keratinocytes in that K8 was present in the HK cell lines but not NHK in cells in submerged culture. A comparison of HK cells and NHK grown at the air-liquid interface revealed a surprising absence of K8 synthesis in HK cell lines suggesting they were moving away from the simple epithelial phenotype. The only difference between HK cells and NHK grown at the air liquid interface was the levels of K6 (higher in HK cells indicative of their higher proliferative capacity. The pattern of keratins in the cell lines is similar to that seen in pre-neoplastic mouse keratinocytes (Yuspa 1991).

All HK cell lines demonstrated a reduced sensitivity to TPA induced loss in colony forming efficiency. 20-25% of HK cell colonies were present after two weeks exposure to TPA, whilst no colonies of NHK were seen when cultured under the same conditions.

Although a direct comparison between NHK and HK cell lines was not determined in this study, HK cell lines did show a significantly reduced ability to form cornified envelopes in the presence of TPA compared with NHK in similar conditions e.g. from morphology experiments (figures 3.7a-d) .

In co-culture experiments HK-2 and HK-4 cells formed colonies amongst differentiating NHK following 14 days exposure to TPA. Although, HK-3 cells demonstrated a growth advantage over NHK cells (i.e. NHK cells were induced to differentiate upon exposure to TPA unlike HK-3 cells) the HK-3 cells did not grow in colonies, rather in evenly spaced groups which were difficult to quantify. This finding further supports the unsuitability of HK-3 cells for the co-culture.

HK-4 cells were chosen over HK-2 cells as the former demonstrated an increased growth rate and approximately 10 % increased resistance to TPA compared with HK-2 cells which should provide them with the capacity to produce more colonies in co-cultures exposed to TPA.

CHAPTER FOUR

Development of an assay for tumour promoters: II. The human cell co-culture model

4.1 INTRODUCTION

The cell culture model of initiated mouse epidermis developed by Hennings (1990) appears to have the potential to predict tumour promoting ability of a diverse range of tumour promoters. It has also been reported to have the ability to measure anti-tumour promoting activity and thus could be useful in discovering agents that can inhibit or prevent the progression of cancer. As yet no such model has been developed to detect promoters in human skin. Nor has there been any conclusive demonstration that human skin undergoes multi-stage carcinogenesis like mouse skin, but unlike skin from most species of hamster (Slaga 1983). In order to determine whether the multi-stage carcinogenesis theory is applicable to human skin, a model of initiated human epidermis was developed. Of the cell lines tested for suitability for inclusion in the co-culture assay, it was concluded in chapter 3 that HK-4 cells were the most representative of initiated human keratinocytes. HK-4 cells were co-cultured amongst a confluent monolayer of normal keratinocytes and the effects of phorbol ester-type and non-phorbol ester-type tumour promoters was assessed. Also studied was the reason why the assay may be calcium-dependant. In the original model, Hennings described the system as being calcium dependent (Hennings 1991), however no indication of why calcium was required was given. As a result in addition the effects of the tumour promoter TPA at various concentrations of calcium was also assessed.

4.1.1 Phorbol ester type-tumour promoters

The phorbol ester-type promoters tested in preliminary studies were TPA, mezerein and sapintoxin A. The tumour promoting activity of TPA and mezerein has been discussed

in chapter 1. Sapintoxin A, a 4-deoxyphorbol ester, has been shown to be a very weak tumour promoter in *in vivo* mouse skin studies (Brooks 1989). It is a potent inflammatory agent and is equipotent to TPA in binding to PKC. The rank order of potency of these drugs as tumour promoters in *in vivo* rodent assays is:

TPA > Mezerein >> Sapintoxin A.

Although the effects of Sapintoxin A and anti-promoters does not appear to have been investigated, both TPA and mezerein can be inhibited by the anti-promoters retinoic acid and fluocinilone acetonide in rodent studies both *in vivo* (Slaga et al 1983*)* and *in vitro* (Hennings 1991).

4.1.2 Non-phorbol ester-type tumour promoters

4.1.2.1 Benzoyl peroxide

Benzoyl peroxide is a widely used free radical generating compound. Its primary use is in the polymer industry although it is also used in preparations for the treatment of acne and as an additive in cosmetics. In recent times caution in the use of benzoyl peroxide has been recommended in view of its tumour promoting ability. It has been reported to promote the formation of papillomas and carcinomas following application of the tumour initiator DMBA in SENCAR mice (Slaga et al 1981). It also shares other properties of tumour promoters in that it produces a marked hyperplasia, stimulates the production of dark basal keratinocytes and inhibits intracellular communication in a dose dependent manner. Furthermore benzoyl peroxide has been reported to be inhibited by anti-promoting agents such as protease inhibitors, retinoids and anti-inflammatory steroids.

The tumour promoting activity of benzoyl peroxide is believed to involve its ability to generate free radicals (see section 1.4.2.4).

4.1.2.2 A23187

The calcium ionophore A23187, also known as calcimycin, has been reported to be a first stage tumour promoter (Fitzgerald & Yamasaki 1992) and also as a co-promoter enhancing the number of papillomas induced by TPA and Sapintoxin A (Brooks 1989). A23187 presumably enhances internal calcium concentration thereby inducing differentiation of normal cells. Since initiated cells have a reduced responsiveness to the induction of terminal differentiation, they would have a growth advantage over the normal cells resulting in the selective clonal expansion of initiated cells. The effects of A23187 as a promoter alone, and also synergistic effects with other promoters was assessed.

4.1.3 Anti-promoters

The ability of this model of initiated epidermis to pick up anti-promoting activity was assessed by incubating the co-culture simultaneously with TPA and the anti-promoters to see if tumour promoter induced clonal expansion could be blocked. Retinoic acid (RA) and the non-steroidal synthetic anti-inflammatory fluocinilone acetonide (FA), have been well documented as antagonists of tumour promotion *in vivo* (Slaga 1980; Verma 1987) and both were reported to inhibit TPA induced colonies in Hennings model of initiated epidermis. Like RA, FA is able to inhibit phorbol ester-and non-phorbol ester-type tumour promoters (Kin & Smart 1995). Both anti-promoters have been tested for their

ability to selectively block the stages of tumour promotion however results have been conflicting. For example Slaga (1980) reported that RA inhibits only stage II tumour promotion in female SENCAR mice, whilst Verma (1987) reported it to block both stages I and II in female SENCAR and CD-1 mice. The controversy in the findings on retinoic acids ability to block either of the two stages of tumour promotion has as yet not been re-evaluated or explained. In order to confirm RAs anti-promoting activity in human studies and also to characterise the model of initiated human epidermis, the ability of RA to inhibit tumour promoter induced colonies was investigated against the stage I promoter TPA and the stage II promoter mezerein. In addition the ability of FA to inhibit clonal expansion was also studied. Finally, both anti-promoters were added together in co-cultures in the presence of TPA to investigate any synergistic or antagonistic effects these anti-promoters may have on inhibition of clonal expansion in the keratinocyte co-culture.

4.2 METHODS

Materials see appendix (i)

Media see appendix (ii)

Co-culture methodology see chapter three

Staining co-cultures see chapter two section (2.2.9)

All experiments were carried out at least 3 times and statistics may be found in appendix.

4.3 RESULTS AND DISCUSSION

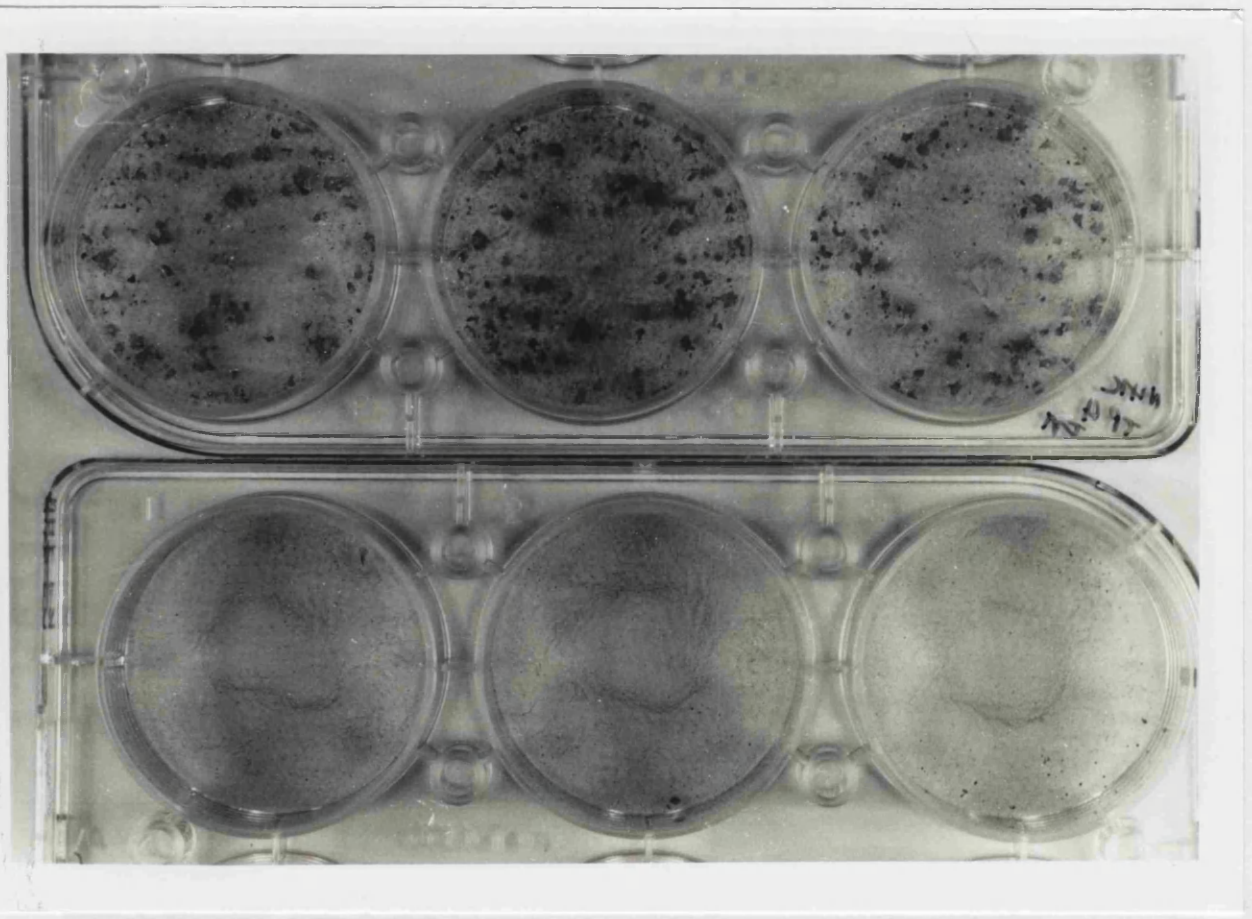
4.3.1 A valid model of initiated epidermis

Co-cultures of approximately 100 HK-4 cells within a confluent layer of NHK were indistinguishable from confluent layers of normal cells alone after 2 weeks in culture. No colonies of HK-4 cells were detected after staining with haematoxylin or immunostaining for keratin 8 using a peroxidase conjugated monoclonal antibody, in the absence of TPA. In the absence of TPA and normal cells, 27.8% ± 2.3 of the HK-4 cells formed colonies after 2 weeks in culture (chapter three). Thus HK-4 cells appear to be inhibited from clonally expanding by the presence of the normal cells. No reduction in clonal expansion of HK-4 cells was seen when keratinocyte conditioned medium was added to HK-4 cells in culture supporting Hennings view that contact between the two cell types is essential.

Continual exposure to 10 nM TPA for 2 weeks dramatically altered the microscopic (figure 4.2d) and macroscopic (Figure 4.1) appearance of the co-cultures compared to controls exposed to 0.01%v/v DMSO. NHK were rapidly induced to differentiate as witnessed by the appearance of squamous and cornified cells. At the end of the exposure period, 25% of the HK-4 cells seeded had formed colonies. In preliminary studies co-cultures were stained with haematoxylin to identify colonies of HK-4 cells (see figure 4.1). Such haematoxylin stained colonies were not seen in cultures of confluent NHK (in the absence of HK-4 cells) exposed to TPA. Microscopic analysis of the colonies suggested not all colonies were HK-4 cell derived. A few colonies were fibroblastic in appearance and were likely to be contaminating 3T3 cells. Therefore in subsequent studies co-cultures were stained with an anti-cytokeratin antibody for K8 which was

found in HK-4 cells but not in NHK (see chapter 3) or 3T3 fibroblasts. Thus colonies were identified as HK-4 cells, and not normal keratinocytes or contaminating 3T3 fibroblasts, because the cells stained strongly for K8 and had a cobblestone morphology. The plating efficiency of HK-4 cells cultured with 3T3 fibroblasts was reduced by approximately 50% in the continual presence of TPA indicating TPA is not mitogenic toward the HK-4 cells (chapter 3, figure 3.6 and see appendix). The HK-4 cells in co-cultures appear to proliferate in response to the space created by the terminally differentiating normal cells. This is supported by the fact that other contact inhibited cells which do not differentiate in response to TPA, e.g. Caco-2 cells, also clonally expanded when co-cultured with a large number of normal keratinocytes in the presence of 10 nM TPA (figure 4.3).

Figure 4.1 *Co-cultures of NHK and HK-4 cells after 14 days incubation either in the presence (above) or absence (below) of TPA stained with haematoxylin*



Colonies of HK-4 cells can be seen in co-cultures exposed to TPA as dark spots. These are absent in co-cultures exposed to DMSO.

Figure 4.2a *Phase contrast photos of co-culture in the presence (i) and absence (ii) of TPA over 14 days* : day 3

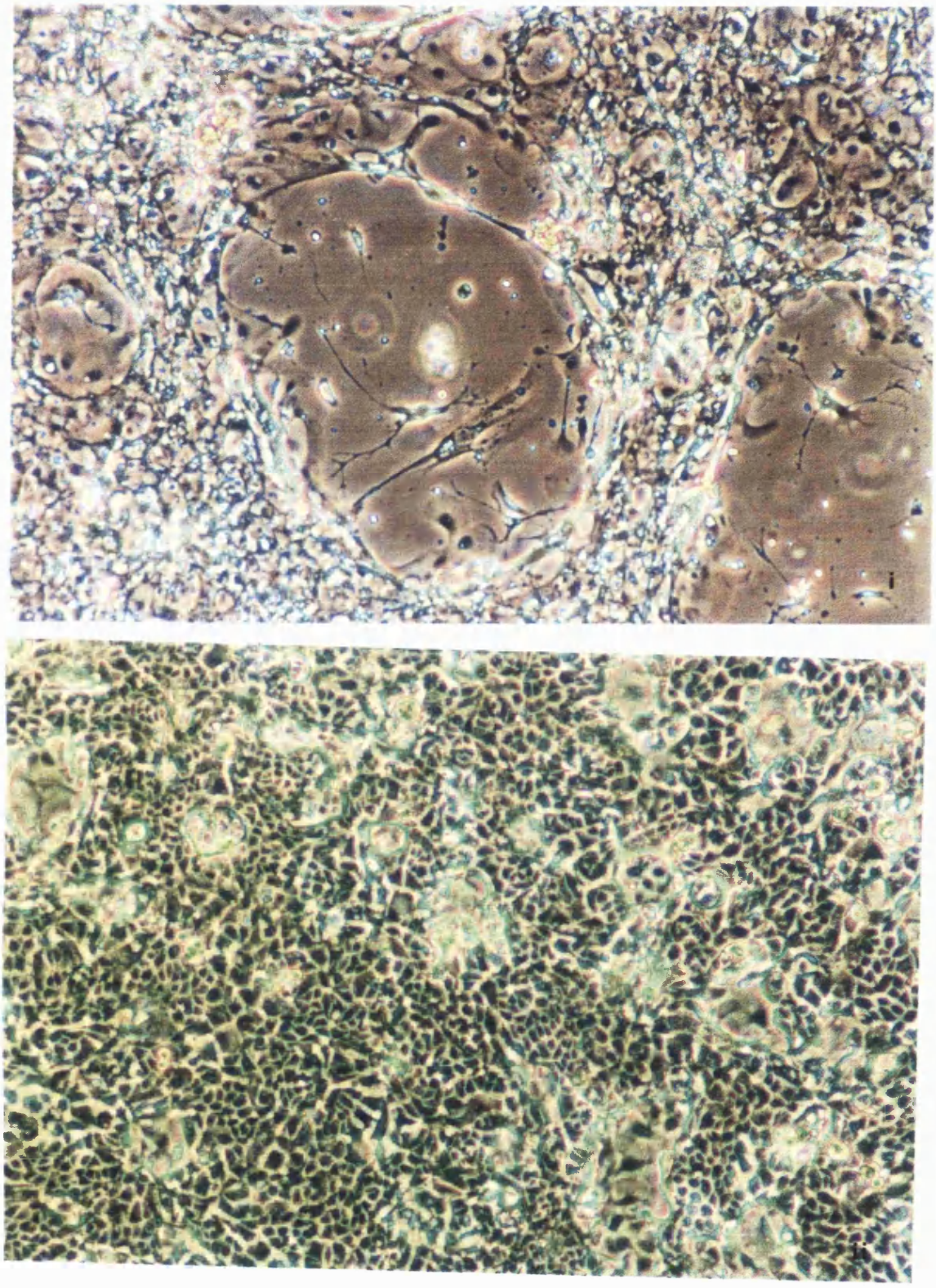


Figure 4.2b *Phase contrast photos of co-culture in the presence (i) and absence (ii) of TPA over 14 days* : day 6

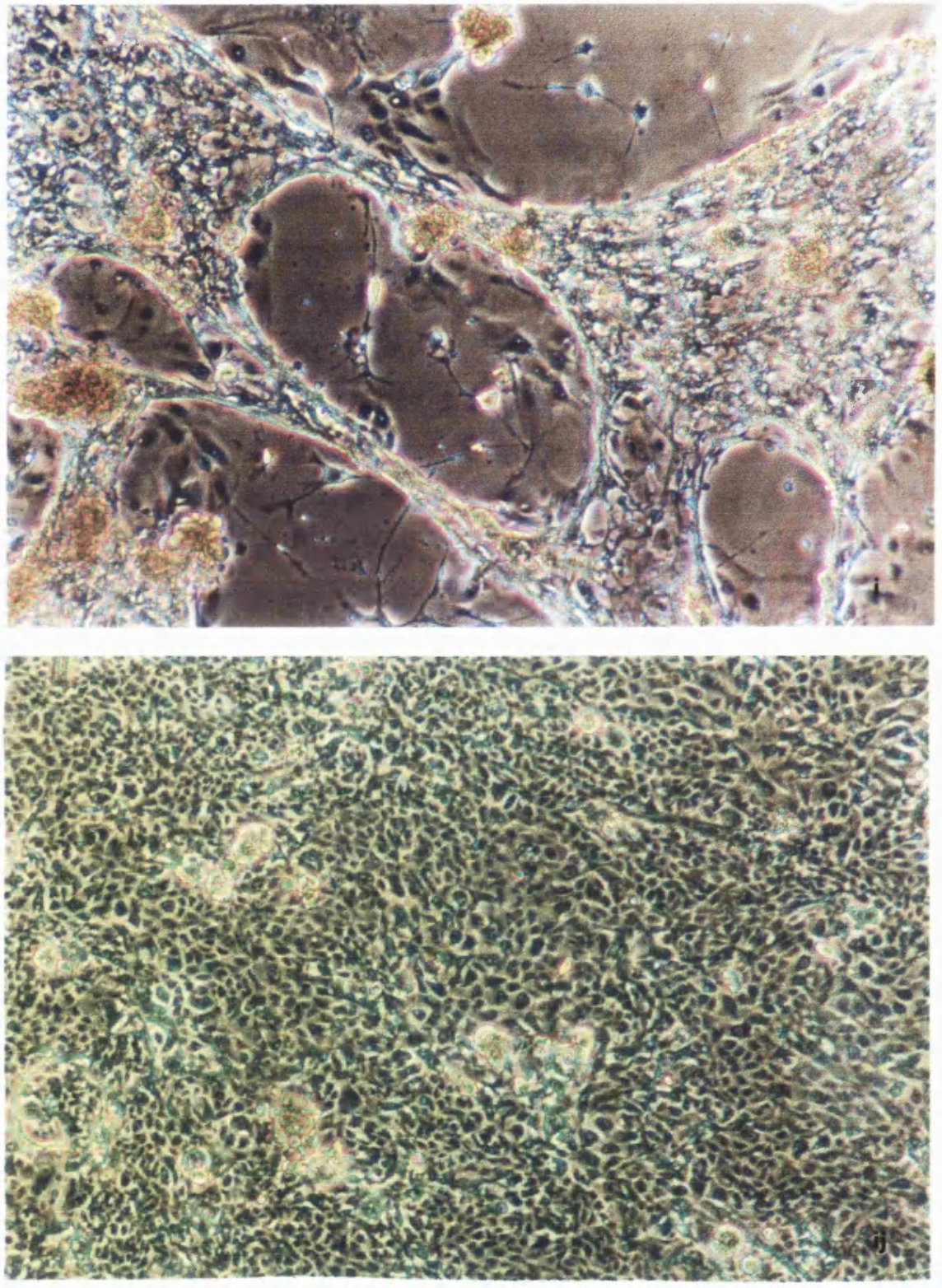


Figure 4.2c *Phase contrast photos of co-culture in the presence (i) and absence (ii) of TPA over 14 days* : day 10

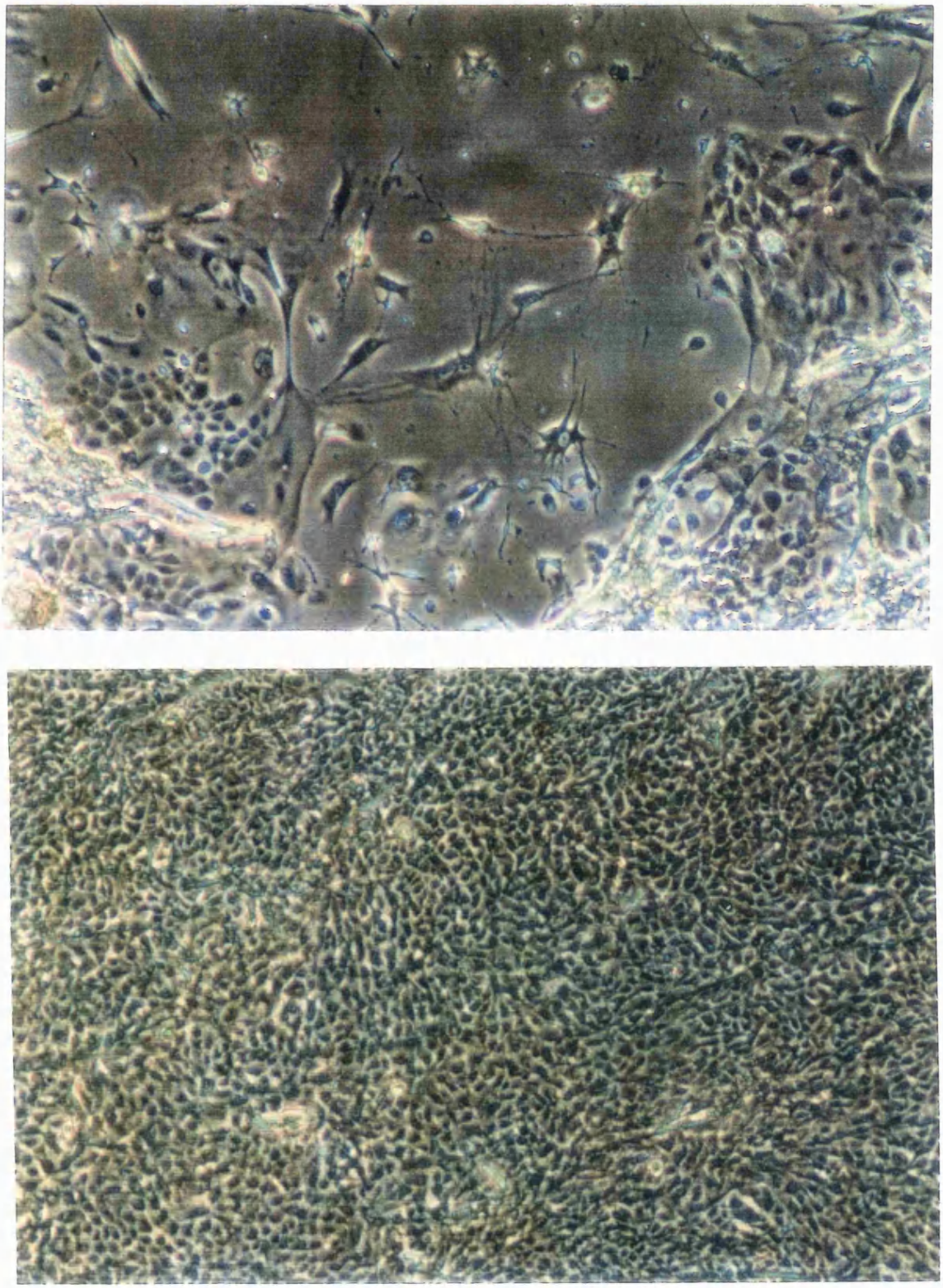
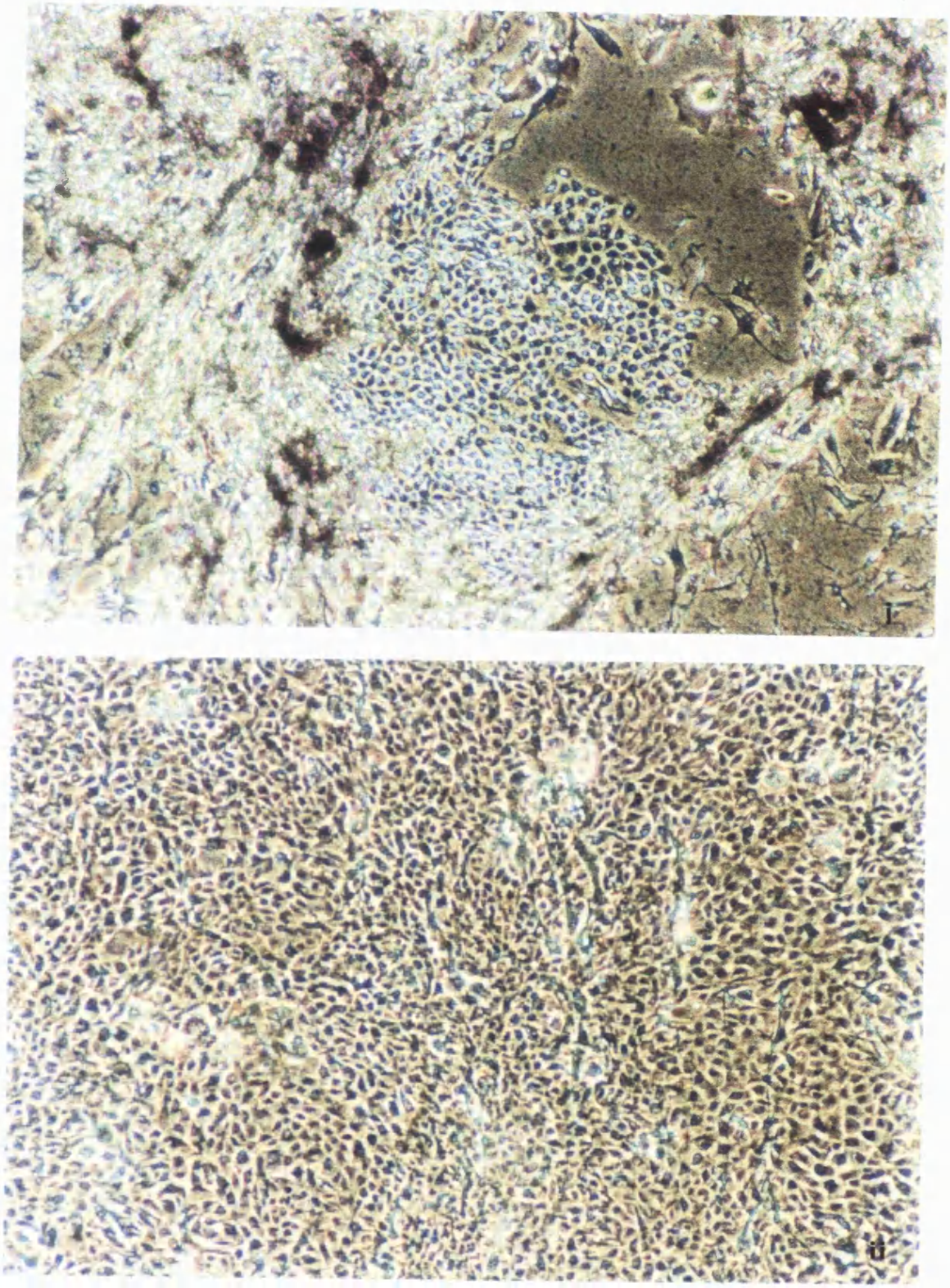


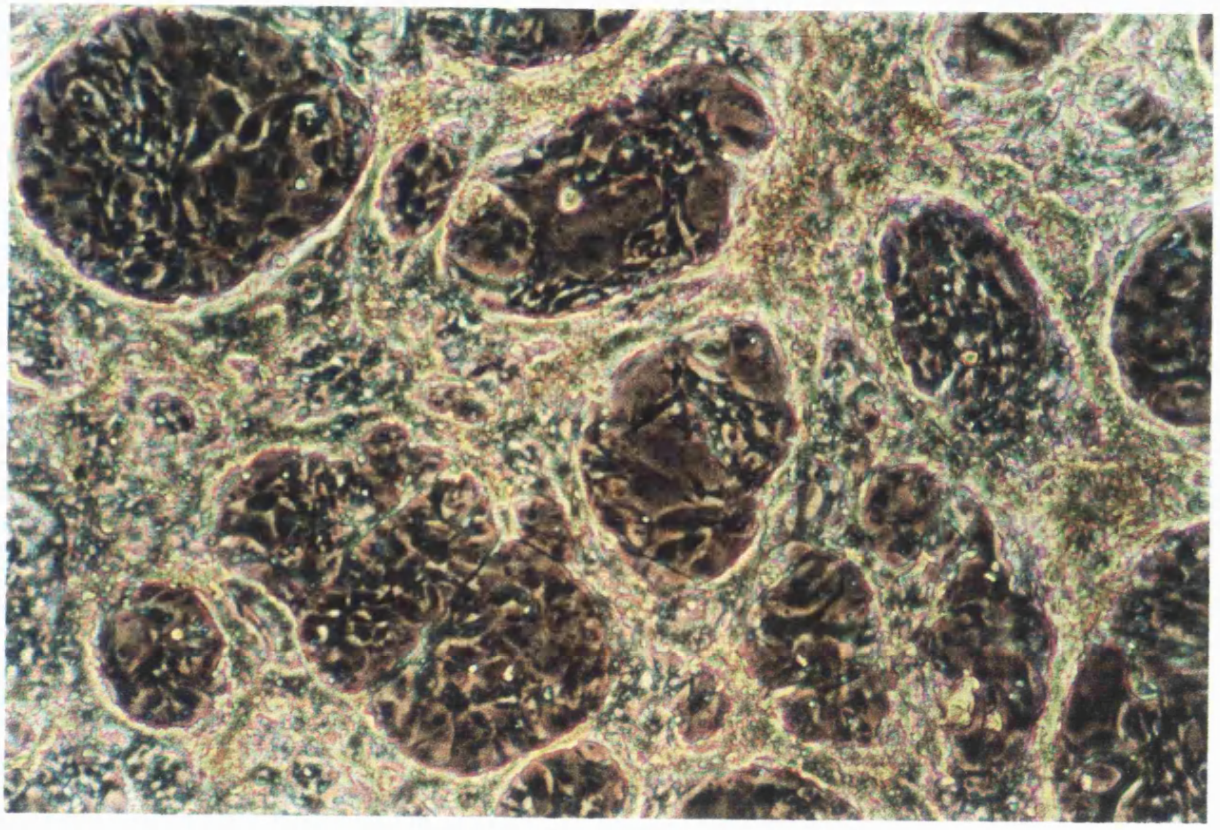
Figure 4.2d *Phase contrast photos of co-culture in the presence (i) and absence (ii) of TPA over 14 days* : day 14



sp= spaces, Dk= differentiating keratinocytes,

Figures 4.2 a-d *depict a temporal sequence of events occurring as a result of exposure of the co-cultures to TPA. Firstly, in the absence of TPA (controls figures 4.2 a-d (ii)) there is relatively little change at the cellular level apart from the overall increase in cell number with time; in these cultures cells became more closely packed together due to normal cell proliferation over the 14 day period. In contrast there appeared to be obvious changes occurring to co-cultures exposed to TPA (figures 4.2 a-d (i)) compared with the controls. At day 1 the co-cultures appeared similar to the controls (not shown), however by day three TPA has induced what appears to be differentiation of the keratinocytes. Differentiating keratinocytes (Dk) appear as extreme bright patches under the confocal microscope, these cells detach from the surface of the flask analogous to cells leaving the basal layer during differentiation. In some areas large areas of the cells had completely detached from the flask leaving spaces (sp). With time the amount of differentiation increased and by day 6 large areas of cell differentiation and detachment became visible. At day 10 colonies of proliferating keratinocytes appeared to grow in the spaces left from detached keratinocytes. These new colonies were later identified as HK-4 cells by positive staining for K8 and also for the large T gene. Furthermore this has been supported by experiments in which confluent NHK alone (i.e. no HK-4 present) were exposed to TPA for 14 days, in these cultures no proliferating colonies were detected.*

Figure 4.3 *Co-culture of Caco-2 cells with NHK cells in the presence of TPA*



Epithelial Caco-2 cells did not respond to TPA induced differentiation. These cells like HK-4 cells, clonally expanded on exposure to TPA when co-cultured with a confluent monolayer of normal keratinocytes. This confirms the suggestion that clonal expansion in the co-cultures is due to an effect of the TPA on the normal cells.

4.3.1.2 Effect of calcium on co-cultures

Two requirements must be met before the model of initiated epidermis is valid. Firstly normal keratinocytes must inhibit the growth of initiated keratinocytes and secondly this must be overcome on exposure to a tumour promoter. The studies carried out on the effects of calcium on the co-culture indicate that in the absence of calcium the former requirement was not being met. The results of growing co-cultures in the presence and absence of TPA in increasing concentrations of calcium are summarised in table 4.1. Colonies of HK-4 cells could be seen in co-cultures grown in media containing 0.09 mM and 0.5 mM calcium, in the absence of TPA (4.4b(i)). Such colonies could not be seen at higher calcium concentrations (1.0 mM and 1.4 mM) (see figure 4.4b(ii)), but did appear when TPA was added (4.4c(ii)). A comparison of the morphology of NHK grown in low calcium medium and high calcium medium shows that at low calcium concentration NHK did not make cell to cell contact, the outline of individual cells can be seen more clearly (figure 4.4a(i)). At the higher concentrations the cells grow in tightly formed colonies with each individual cell making contact with neighbouring cells (see figure 4.4a(ii)). Presumably the lack of cell to cell contact at low calcium concentrations prevents the NHK from making contact with HK-4 cells and therefore prevents inhibition of growth. As the calcium concentration was increased, cells made contact and HK-4 cells were prevented from clonally expanding. Thus the model is invalid at low calcium concentrations as NHK do not inhibit the clonal expansion of HK-4 cells due to a lack of cellular contact and as a result colonies are seen both in the presence and absence of tumour promoters (4.4b(i) and 4.4c(i)). These results support Hennings findings that high calcium concentration and cell contact are essential requirements in this model of initiated epidermis. In order to confirm and quantitate the importance of cell to cell contact GJIC could be measured and compared between co-cultures grown in high and

Table 4.1. The presence of HK-4 colonies in co-cultures in the presence and absence of 10 nM TPA grown in serum-free medium with increasing concentrations of calcium

[Calcium]	Without	With TPA
0.09 mM	✓	✓
0.50 mM	✓	✓
1.0 mM	✗	✓
1.4 mM	✗	✓

Figure 4.4(a) *NHK grown in low 0.09 mM (i) and high 1.4 mM (ii) calcium media*

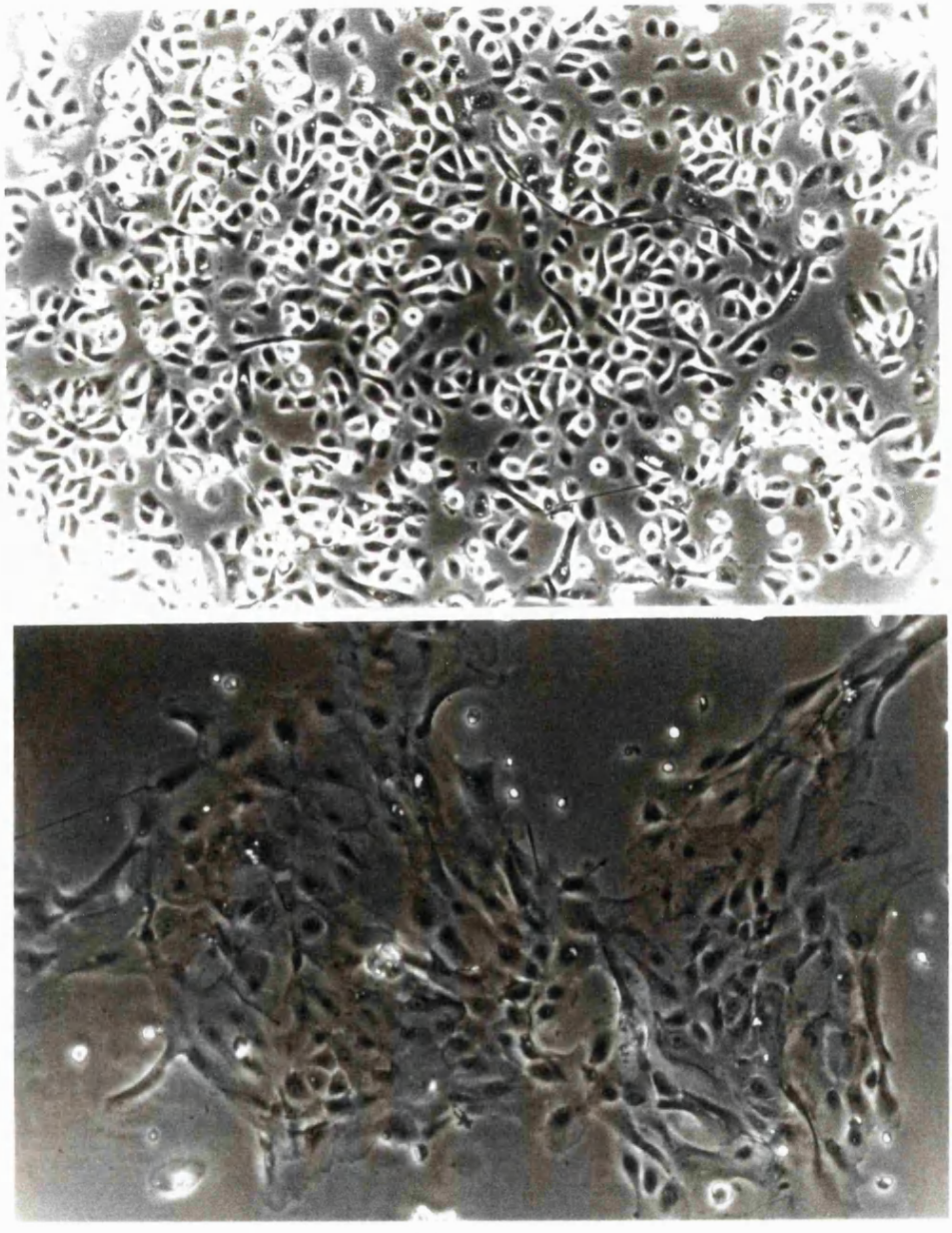


Figure 4.4(b) *The effects of calcium on co-cultures grown in serum free medium in the absence of TPA : (i) 0.5 mM calcium and (ii) 1.4 mM calcium*

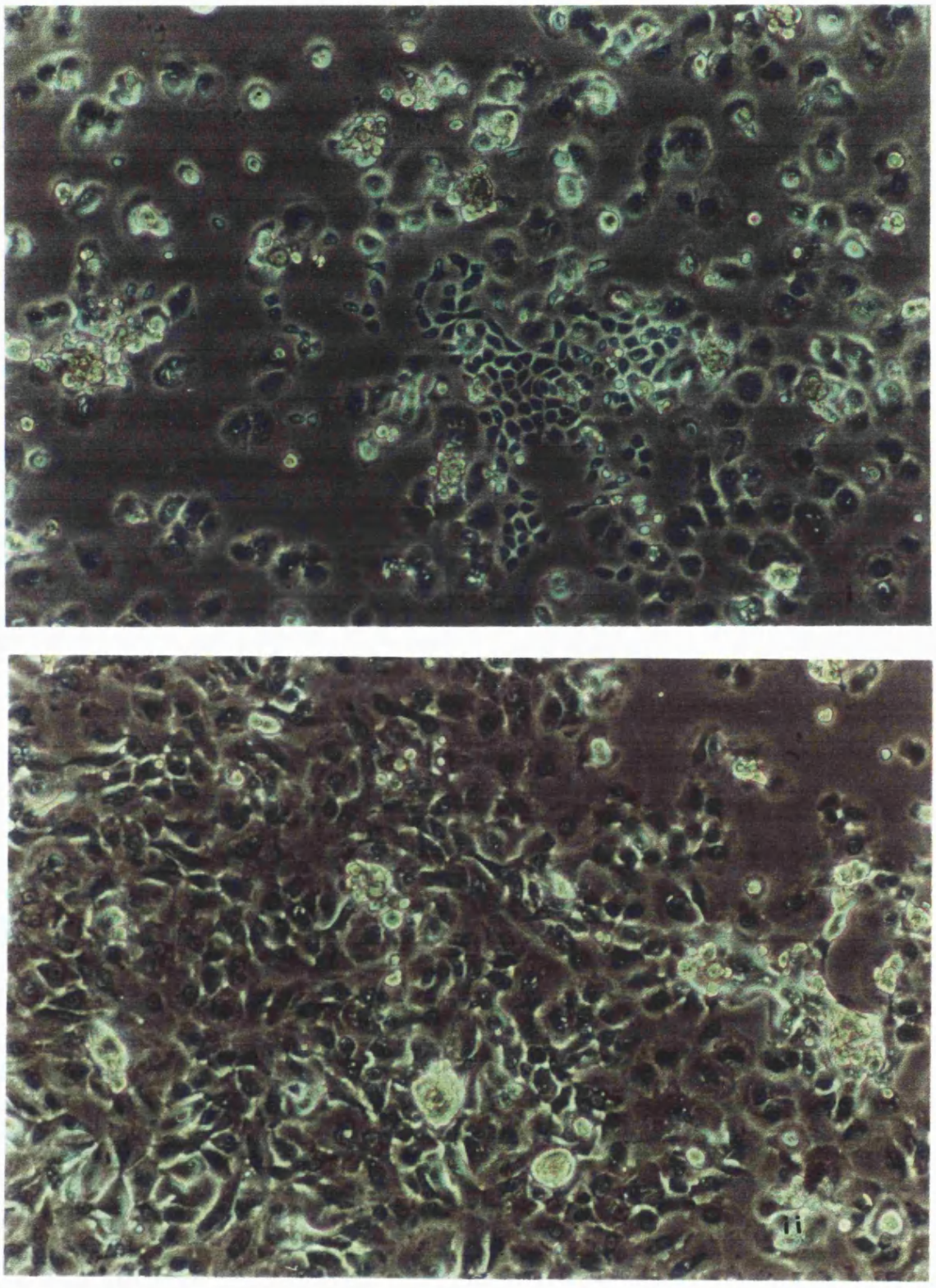
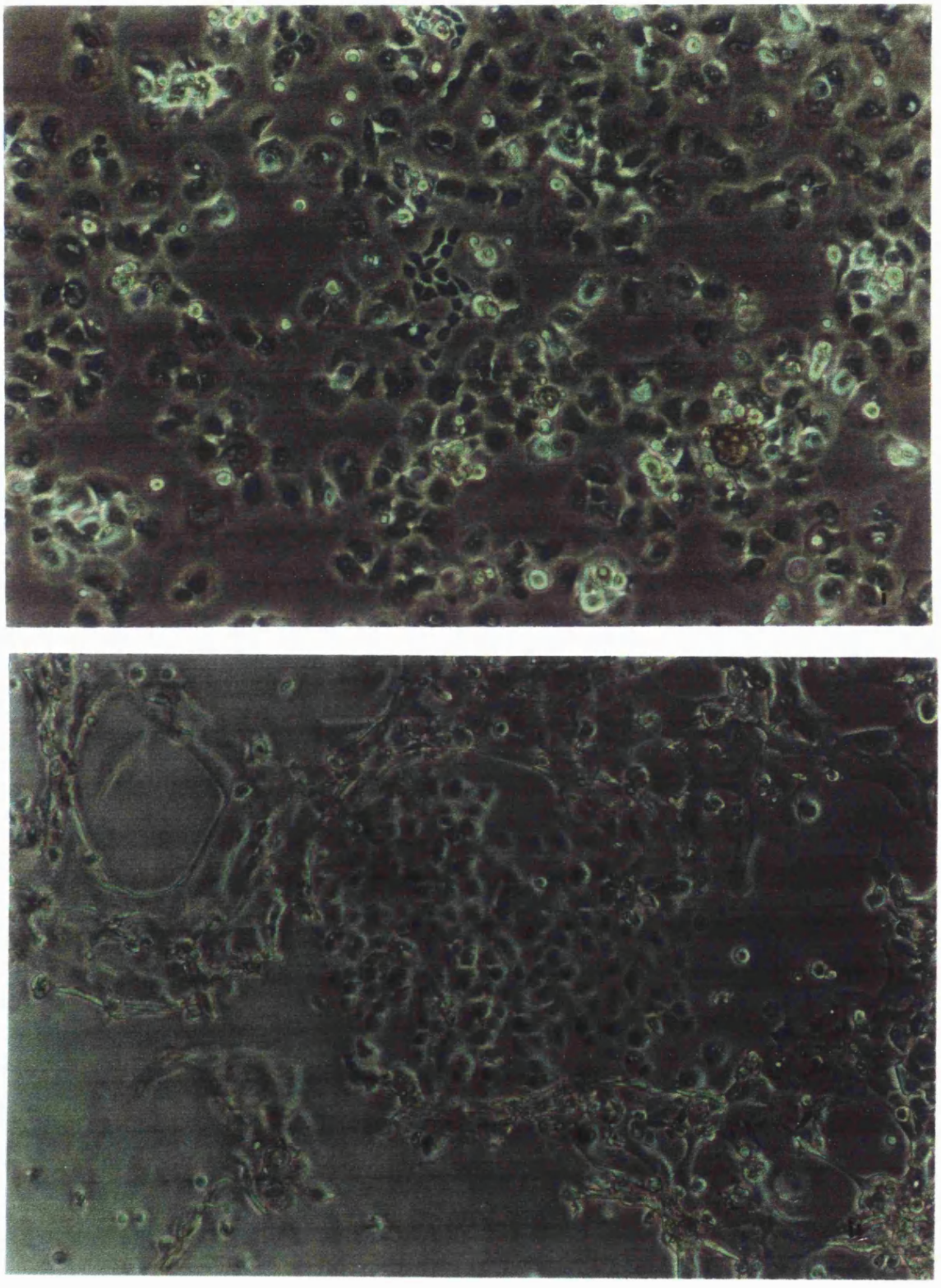


Figure 4.4(c) *The effects of calcium on co-cultures grown in serum free medium in the presence of TPA : (*i*) 0.5 mM calcium and (*ii*) 1.4 mM calcium*



4.3.2 Blind assay

As an initial test of the potential of this system as an assay for skin tumour promoters, a blind assay study was instigated using TPA (a), mezerein (b), sapintoxin A (c) and a blank (d). A double blind assay was conducted to reduce any bias in measurements that may have arisen otherwise. These samples were kindly prepared by Jon Ryves from the pharmacognosy department at the School of Pharmacy. Co-cultures were exposed to each sample (10 nM) for 2 weeks alongside positive and negative controls (10 nM TPA and 0.01% DMSO) after which they were fixed and immunostained for keratin 8. Brown stained colonies were measured and counted.

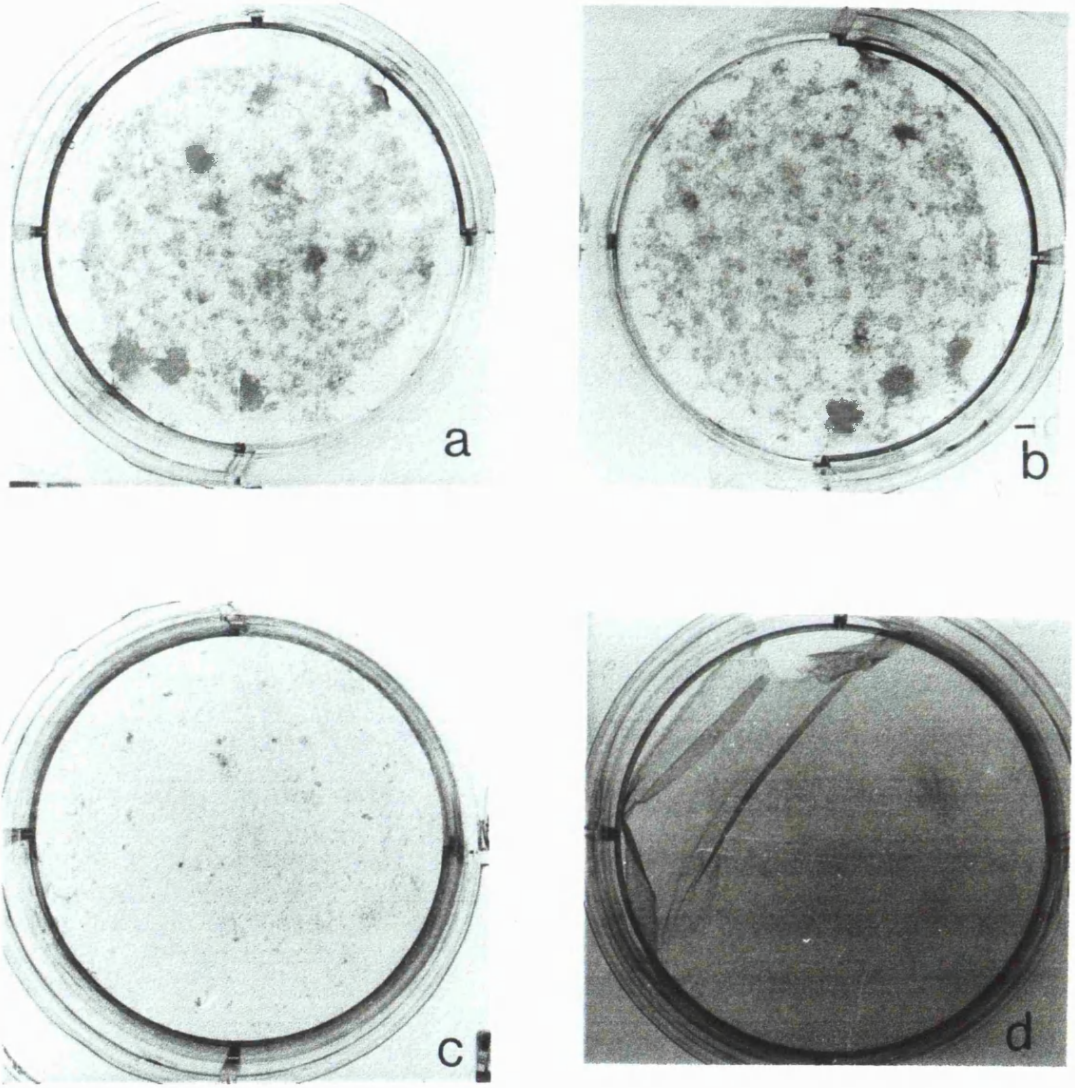
Compound D was correctly identified as the blank as, like the negative control a confluent layer of keratinocytes remained after 2 weeks in culture and no HK-4 colonies were detectable after staining for K8. Compound A was identified as TPA because the co-cultures were identical to the positive control in that a similar number of large colonies of HK-4 cells appeared after 2 weeks exposure to the test chemical (figure 4.5a).

Co-cultures exposed to compound B were similar to the positive control with large spaces appearing in the cell layer and a similar number of colonies emerging. However the colonies were markedly smaller than those produced by TPA (figure 4.5c). Co-cultures exposed to compound C closely resembled the negative control with a confluent monolayer of keratinocytes remaining after two weeks culture. Upon staining for K8 however, small colonies (1 mm diameter) of HK-4 cells could be identified (figure). On the assumption that tumour promoter potency would be mirrored by the size of the HK-4

colonies produced, compounds B and C were correctly identified as being mezerein and sapintoxin A respectively.

In quantifying the effects of tumour promoters and anti-promoters in the mouse model of initiated epidermis, Hennings counted the number of colonies of 308 cells. After carrying out the blind assay and also dose response curves for the promoters, it became apparent that the size of the colonies increased with increasing dose of promoter and also appeared to reflect the potency of the promoter. Therefore in subsequent experiments the size of the largest 5 or 10 colonies were measured and used to quantify effects of promoters on the clonal expansion of HK-4 cells.

Figure 4.5 *Effects of incubating the co-culture in the presence of (a) TPA, (b) Mezerein and (c) Sapintoxin A at 10 nM and (d) 0.01% v/v DMSO control.*



Colonies of HK-4 cells as identified by positive staining with an antibody for cytokeratin 8 can be seen in figures a, b and c. Those in figures a and b are of a similar size whilst those in figure c are significantly smaller. No colonies could be identified in co-cultures exposed to 0.1% DMSO. The largest five colonies in each plate were measured and the sum of 3-5 individual experiments was used to quantify the data.

Figure 4.6 *Dose response curves of phorbol ester-type tumour promoters*

Data shown are means and standard errors, n=3-5. See appendix for statistics.

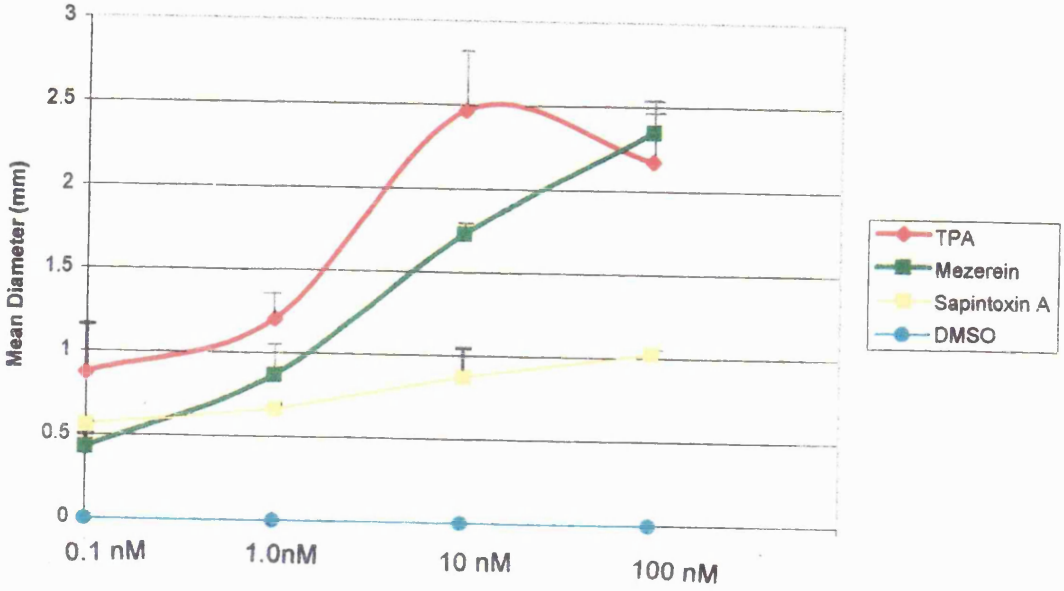
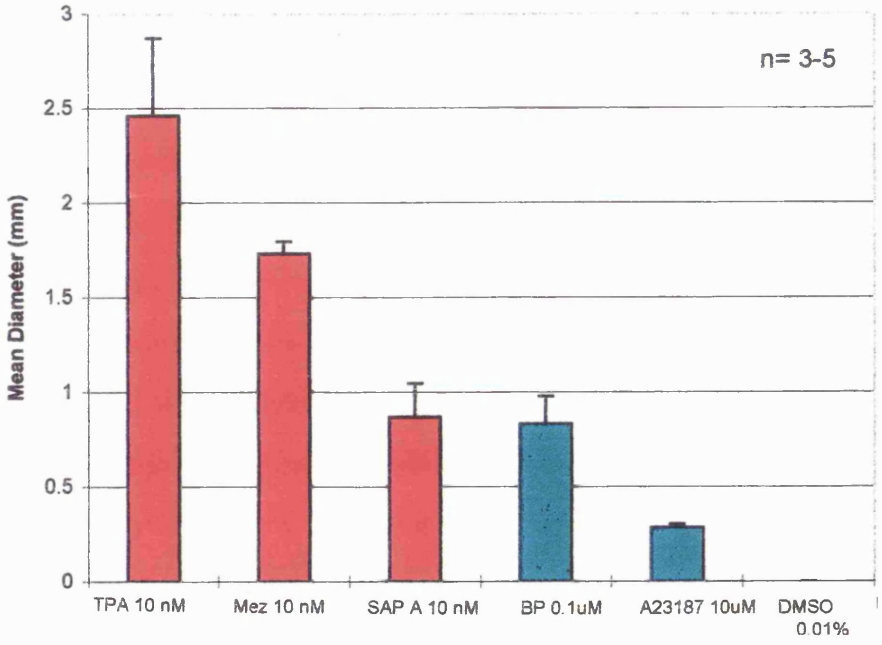


Figure 4.7 *Average diameter of colonies of HK-4 cells exposed to phorbol ester- and non-phorbol ester-type tumour promoters for 14 days.*



4.3.3 Non-phorbol ester-type tumour promoters

The effects of the non-phorbol ester-type promoters benzoyl peroxide and A23187, were assessed for their ability to induce clonal expansion of HK-4 cells in co-cultures using the same method as for phorbol ester-type promoters. Both benzoyl peroxide and A23187 induced the clonal expansion of HK-4 cells in co-cultures after 14 days of continuous exposure.

Benzoyl peroxide (10^{-5} M) was a very weak promoter inducing colonies of HK-4 cells of approximately 0.8 mm mean diameter. At 10^{-4} M benzoyl peroxide produced fewer but similar sized colonies. At concentrations below 10^{-5} M benzoyl peroxide, no colonies were seen. A23187 (10 μM) induced a similar size of colony however fewer colonies in these co-cultures brought the mean diameter down. Clearly both the size and the number of colonies as well as the concentration of the promoter need to be taken into account in quantifying potency. Higher doses of A23187 were tested, however these caused the monolayer of cells to detach from the culture as a sheet and therefore it was impossible to measure clonal expansion at doses above 10 μM. Similar effects were seen when 10 μM. A23187 was added simultaneously with 10 nM TPA.

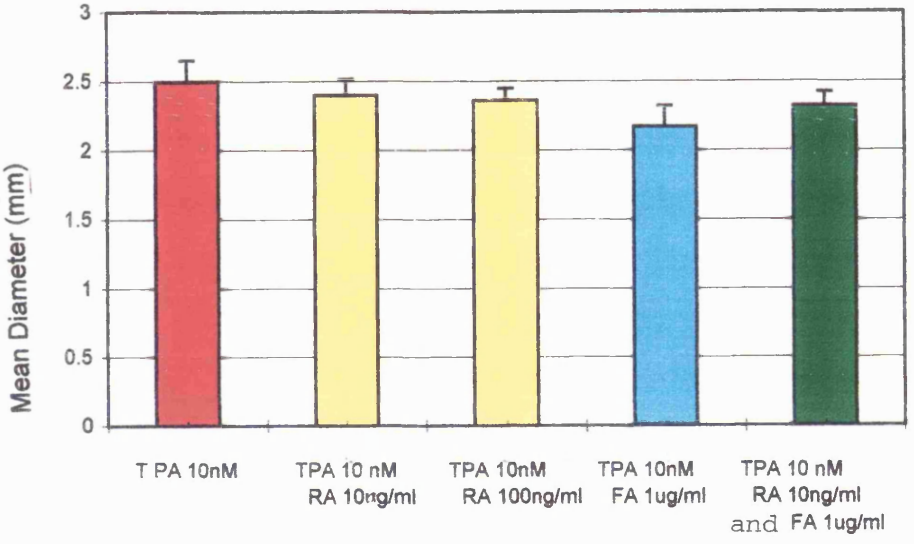
4.3.4 Anti-promoters

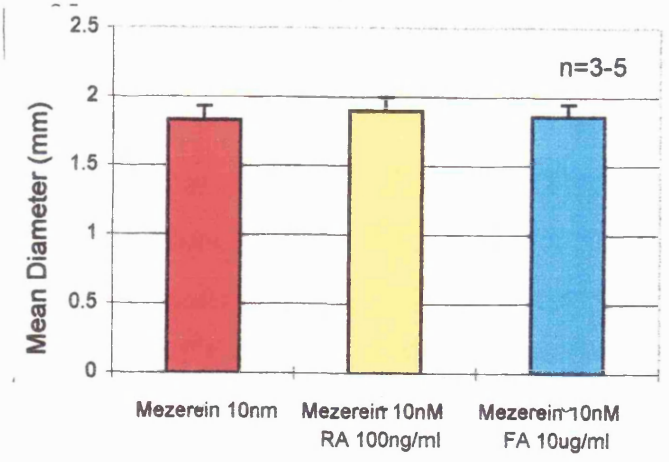
When RA or FA were added to co-cultures simultaneously with TPA or mezerein, no significant decrease in either the size or number of colonies was apparent (see figure 4.8). Thus although like Hennings model this model was able to identify tumour promoters, both phorbol ester and non-phorbol ester-type, our model unable to detect anti-promoting

activity of RA (1-100 ng/ml) or FA (0.1-10 μg/ml). A number of possibilities for this anomaly have been considered.

Figure 4.8 *Effects of anti promoters on clonal expansion of HK-4 cells in a human model of initiated epidermis*

Data shown are means and standard errors, n=3-5. See appendix for statistical analysis.





4.3.4.1 Anti-promoters: Species differences

It is possible that the results that have been indicated i.e. that retinoic acid and fluocinilone acetonide inhibit promotion in mouse keratinocytes but not human keratinocytes are correct. Species differences in response to retinoids have been discussed in chapter 1). However, studies on humans have shown retinoic acid to reduce cancers of the skin (Hill and Grubbs 1992 : Kligman & Thorne 1991)

4.3.4.2 Anti-promoters: *in vivo* to *in vitro* differences

The mechanisms underlying the clonal expansion assay may be summarised as follows:

1. Initiated cells are prevented from growing by the surrounding normal cells.
2. TPA induces the normal cells to differentiate. Thus contact between HK-4 and NHK cells is lost as the latter cell type sloughs off the culture dish. As a result HK-4 colonies grow at the expense of NHK. Presumably anti-promoters somehow prevent TPA induced differentiation of NHK and thus also prevent TPA induced clonal expansion.

Hennings model of initiated mouse epidermis involves replacing NHK that were differentiating in response to TPA, with fresh primary keratinocytes. He reported that when keratinocytes were added only at time of plating, neither retinoic acid or fluocinilone acetonide inhibited TPA induced 308 formation (Hennings 1990). It may be suggested that the second administration of NHK alone could have prevented the growth of 308 cells rather than the anti-promoters.

Further evidence to support the suggestion that RA may not have activity in these monolayer co-cultures comes from a study that investigated the levels of CRABP II. This RA receptor appears to modulate anti-differentiation/proliferation of keratinocytes and therefore possibly the anti-promoting effects of RA (chapter 1 section 1.5.4.2). On comparing levels of CRABP II in normal skin, submerged keratinocyte cultures and human skin equivalent it was shown that CRABP II was not induced in keratinocytes in submerged culture yet a clear induction was seen in intact human skin and in skin equivalents. This suggests that the induction of CRABP II by RA requires epidermal stratification and/ or dermal-epidermal interaction (Elder et al 1992). If RA modulates its effects via CRABP II, then the anti-promoting effects of RA would not be seen in the submerged co-cultures. Since CRABP II is induced in keratinocytes in a skin equivalent, it may be of benefit to grow the co-culture on a dermal equivalent and monitor the effects of promoters and anti-promoters in this model of initiated skin.

4.3.4.3 Anti-promoters: Timing of application

Verma (1977) reported that the timing of application of RA was important in anti-promoting activity. RA needs to be applied before and during promoter exposure for maximum effects. In this study and also in the study carried out by Hennings, anti-promoters were added simultaneously with promoters. Application of anti-promoters at least 24 hours prior to, and also concurrently with promoters may allow the detection of anti-promoting activity in this model.

4.4 CONCLUSIONS

The co-culture of HK-4 and NHK as a model of initiated epidermis was able to identify TPA, mezerein and sapintoxin A in a dose dependant manner and rank them in order of potency according to the average size of the largest 5 HK-4 colonies. The ranking matched the order of potency of these samples as skin tumour promoters in mouse skin. The model is therefore able to identify both stage I (TPA) and stage II (mezerein) phorbol ester-type tumour promoters. The clonal expansion of HK-4 cells does not reflect the ability of these promoters to bind PKC since TPA and sapintoxin A which exhibit equipotent PKC binding affinities, produce different size colonies of HK-4 cells; Colonies of HK-4 cells were much larger in TPA treated co-cultures compared with the weaker promoter Sapintoxin A. The co-culture is also able to identify the non-phorbol ester-type stage I promoters A23187 and benzoyl peroxide.

Although the anti-promoters RA and FA were reported to inhibit clonal expansion in the mouse model of initiated epidermis, no inhibition of clonal expansion was seen in human co-cultures when exposed concurrently to TPA or mezerein and RA or FA, compared with exposing co-cultures to the promoter alone. This may be attributed to experimental error in the timing of anti-promoter application or due to *in vitro* to *in vivo* differences as a result of the absence of crucial molecules modulating the effect of anti-promoters e.g. CRABP II.

CHAPTER FIVE

Development of an assay for tumour promoters:

III. A three dimensional model of human initiated epidermis

5.1 INTRODUCTION

The submerged co-culture of initiated human epidermis described in chapter three, appeared to be successful in identifying both phorbol ester type and non-phorbol ester-type tumour promoters but not anti-promoters. One of the reasons behind this was attributed to morphological and biochemical differences in keratinocytes in submerged cultures compared with normal skin. For example, an essential protein which is believed to be required for the action of retinoic acid, CRABP II, appears to be absent or at a very low concentration in cultures in monolayer. This enzyme has however been identified in skin equivalents. Other examples of biochemicals present both in skin *in vivo* and skin equivalents *in vitro* but not in submerged cultures have been discussed in chapters 1 and 2.

The mechanism of action of the promoters and anti-promoters appears to involve alterations to the normal pattern of growth and differentiation. Initiated cells are hyperproliferative and show a reluctancy to differentiate under conditions normal cells would be induced to differentiate e.g. in the presence of TPA. This growth advantage plays an integral part in allowing the selective growth of initiated cells over normal cells resulting in tumours in conditions favouring differentiation. Therefore in studying carcinogenesis *in vitro* it is essential that both types of keratinocyte (initiated and normal) exhibit their normal patterns of differentiation. Growth and differentiation patterns of normal keratinocytes cultured in monolayer have been reported to be far removed from normal cells *in vivo* as shown by the absence or low levels of markers of differentiation such as keratins K1 and K10, keratohyalin granules, filaggrin and cornified envelopes

(discussed in chapter 2). Keratinocytes grown at an air-liquid interface in organotypic culture appear to resemble skin *in vivo* more closely both structurally and biochemically. Therefore this type of model would be more suitable for studying disorders of differentiation such as cancer.

Compounds tested in submerged co-cultures require solubilisation in culture medium. This restricts the testing of water insoluble compounds. A large number of environmental and occupational pollutants are insoluble organic compounds and the route of exposure to these compounds is most often absorption through the skin. An organotypic model of skin (similar to that described in chapter 2), which incorporated the co-culture (chapter 4), would allow the direct application of water insoluble compounds on the epithelial layer analogous to a topical application whilst the maximum effects of water soluble compounds could be tested by dissolving such compounds in the culture medium below the 3D model of initiated epidermis. This would not only increase the number of compounds that may be tested, but also provides a choice in the route of administration which may be important since the route of administration of compounds can affect their tumour promoting ability (Hill & Grubbs 1991: Silverman et al 1981)

Due to time constraints this chapter was limited to identifying HK-4 cells in a co-culture of HK-4 and NHK cells cultivated at an air-liquid interface on contracted collagen fibroblast lattices. K8 was used to identify HK-4 cells in a co-culture of NHK and HK-4 cells in monolayer, however electrophoresis studies comparing keratin patterns in cells in monolayer and at the air-liquid interface have indicated a reduction of K8 synthesis by

HK-4 cells cultivated at the air-liquid interface (chapter 3). Thus this biomarker would not be suitable for identifying clonal expansion of HK-4 cells in the three dimensional model of human initiated epidermis. Although levels of K6 appeared to be high in HK-4 cells on the skin equivalent, whilst levels in NHK appeared to be insignificant, this keratin was also regarded as inappropriate for identifying HK-4 cells as it is not completely exclusive to the transformed cells. Thus keratins cannot be used to distinguish between the two cell types in organotypic culture. HK-4 cells have been transformed using a sub-genomic SV40 gene which inserts DNA, encoding for the large T gene. Thus staining for the large T antigen may prove to be the most appropriate method for identifying HK-4 cells. Immunohistochemical staining using an antibody for the large T gene on skin equivalents made up of increasing ratios of NHK:HK-4 cells in organotypic culture to investigate whether the HK-4 cells could be distinguished from NHK.

5.2 METHODS

Materials see Appendix (i)

Media see Appendix (ii)

NHK and HK-4 cell were cultured as described in Chapter 2 section 2.2.2.

Histology was carried out as described in Chapter 2 section 2.2.6.

Immunohistochemical staining was carried out as described in chapter 2 section 2.2.9.

5.2.1 Setting up co-cultures on the dermal equivalent

Dermal equivalents were set up as described in chapter 2 (section 2.2.3). On day 7, NHK and/or HK-4 cells were seeded into stainless steel wells onto the dermal equivalents (see Table 5.1 for quantities). The wells were removed 4 hours after seeding and the skin equivalents cultivated immersed in Skin equivalent medium for one week before raising to the air-liquid interface. Medium was changed every other day.

Table 5.1 *The number of NHK and HK-4 cells seeded onto dermal equivalents*

	NHK	HK-4
100% NHK	5×10^5	0
75% NHK + 25% HK-4	3.75×10^5	1.25×10^5
50% NHK + 50% HK-4	2.5×10^5	2.5×10^5
25% NHK + 75% HK-4	1.25×10^5	3.75×10^5
100% HK-4	0	5×10^5

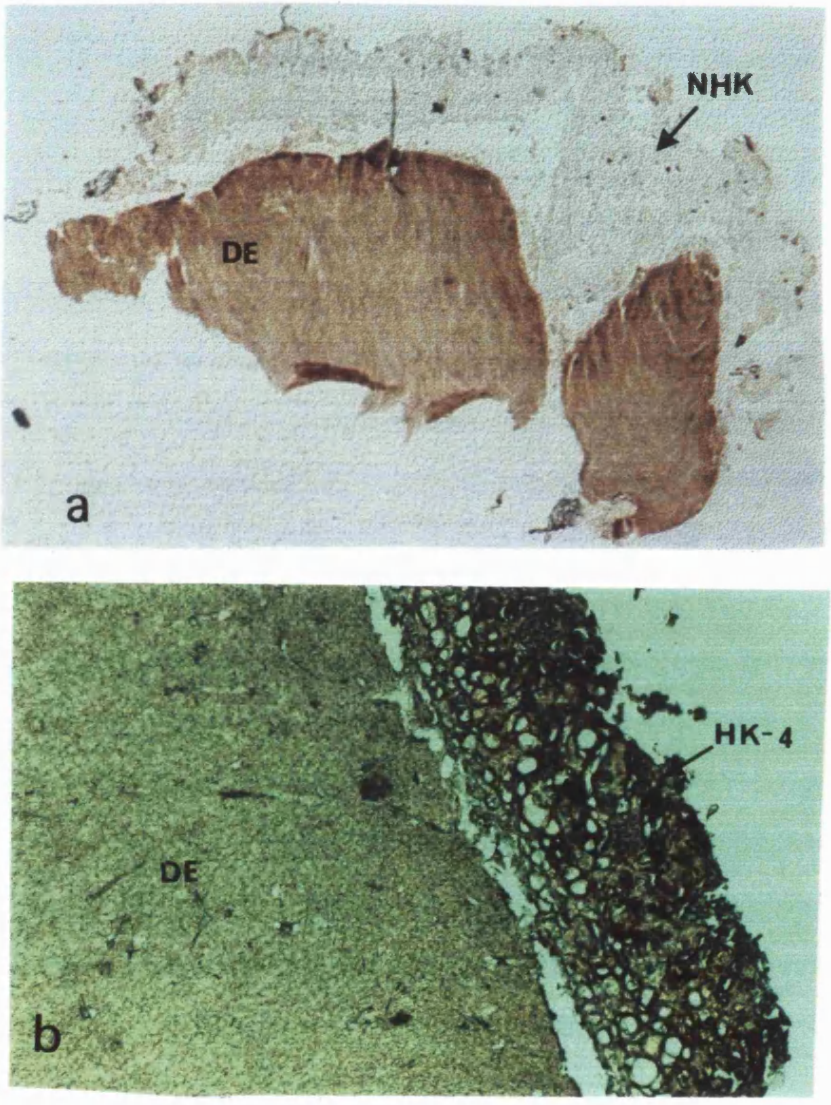
5.3 RESULTS AND DISCUSSION

NHK and HK-4 cells could be distinguished on the skin equivalent after staining for the large T antigen (figure 5.1). However no increase in the intensity of the stain was seen upon increasing the proportion of HK-4 cells on the skin equivalent co-cultures. The ability to monitor an increase in the number of cells is fundamental to assessing tumour promotion in this system. The technique of immunohistochemistry did not appear sensitive enough to detect increasing numbers of HK-4 cells. This may be because HK-4 cells are evenly spread throughout the epithelial layer. Also HK-4 cells have a faster growth rate than NHK and although a specified number of cells were seeded onto dermal equivalents, they were subsequently allowed to grow on the dermal equivalent for 3 weeks prior to processing, therefore the ratio of NHK to HK-4 cells may have been significantly altered by the time samples were stained. In the working model however, HK-4 cells are expected to grow in small tight colonies in the presence of tumour promoters and these should be more easily identified amongst the surrounding non-staining NHK.

Clearly this system requires further investigation. Co-cultures of a few (10-100) HK-4 cells surrounded by 4×10^5 NHK, and grown on a dermal equivalent, need to be stained after 2 weeks exposure to TPA to determine whether clonal expansion can be identified by immunohistochemical techniques staining for the large T antigen. There is a possibility that colonies of HK-4 cells could be too small after only 2 weeks exposure and would therefore be missed if insufficient samples are taken during the histology process. Thus as *in vivo* rodent studies this model would require some histological expertise and

may be fairly time consuming compared with the submerged co-cultures described in chapter 4.

Figure 5.1 *NHK (a) and HK-4 (b) cells grown on dermal equivalents at the air liquid interface and stained for large T antigen*



Photographs show histological horizontal sections of skin equivalents made up of either NHK (a) or HK-4 cells (b), stained for the large T antigen using diaminobenzidine staining. NHK grown on a dermal equivalent do not stain for the large T gene (note the absence of stain in the cells). Whilst HK-4 cells are stained intensely.

5.4 CONCLUSIONS

The antibody for the large T antigen was able to distinguish between normal and transformed cells grown on a dermal equivalent at the air-liquid interface. Experiments to investigate the effects of identifying increasing ratios of HK-4 to NHK cells in homogenous co-cultures showed no increase in the intensity of the stain. These experiments did not represent the clonal expansion of a few HK-4 cell colonies surrounded by normal cells that would be expected following exposure of the three dimensional model of initiated epidermis to tumour promoters. Further experiments need to be carried out to verify the applicability of this technique in assessing and quantifying clonal expansion of HK-4 cells in the organotypic model.

CHAPTER SIX

General Discussion

6.1 Introduction

Hennings (1990, 1991, 1992) has described what appears to be a successful model of initiated mouse epidermis by co-culturing initiated and normal keratinocytes as a submerged monolayer. This model has been reported to successfully identify a diverse range of promoters and anti-promoters. This thesis has described the development of a similar model of human initiated epidermis by producing a co-culture comprising a few transformed keratinocytes (HK-4) amongst a confluent monolayer of normal keratinocytes. In this system tumour promoters induced the clonal expansion of HK-4 cells by selectively stimulating the differentiation of the normal cells. Although able to identify tumour promoting agents it did not identify anti-promoters. Since test compounds need to be dissolved in culture medium, the model was not useful in the testing of water insoluble compounds.

Also, a three dimensional model of skin has been set up using readily available sources. Like other models of reconstituted skin it resembles the epidermis *in vivo* more closely both morphologically and biochemically and therefore would be expected to imitate responses of the human skin *in vivo* more accurately than the submerged monolayers.

The co-culture model of initiated epidermis was developed by incorporating the co-culture within a three dimensional model of human skin to form a stratified initiated epithelium at the air-liquid interface. This organotypic model should increase the number of chemicals that could be tested for promoting activity, as water insoluble compounds could be applied directly on the epidermis whilst water soluble compounds could

continue to be dissolved in culture medium. Both models have the potential to identify tumour promoters and anti-promoter activity. A summary of the findings of this study and recommendations for future work are discussed in this final chapter.

6.2 A model of initiated human epidermis: co-culture as a submerged monolayer

6.2.1 The effect of tumour promoters

The co-culture of HK-4 and NHK cells as a model of initiated epidermis appears to be a good system for identifying both phorbol ester-type (TPA, mezerein and sapintoxin A) and non-phorbol ester-type (A23187 and benzoyl peroxide) promoters. However more tumour promoters need to be tested in this model, in particular tumour promoters with different mechanisms of action e.g. okadaic acid, staurosporin and teleocidin, and also non-tumour promoting agents e.g. resiniferatoxin and α-TPA, before it can be accepted as a model for identifying tumour promoters.

Synergism between tumour promoters and the applicability of two stage promotion also needs to be investigated. Although the stage II promoter, mezerein, was identified in the submerged co-cultures, no attempt was made to simulate two stage tumour promotion in the model. It would be interesting to learn whether or not the two stage tumour promotion protocol could be applied to the co-culture *in vitro*. Also, synergistic effects of promoters, although investigated with TPA and A23187, could not be measured as the calcium ionophore induced peeling of the epithelial layer from the culture plate. The effects of TPA and other tumour promoters should be tested and compared with results from *in vivo* studies to identify the possibility of synergism between promoters.

The current method of measuring the diameter of the largest 5-10 colonies is a very inaccurate and subjective method for quantifying the potency of tumour promoters. A computer assisted optical determination of HK-4 colony size could be performed by scanning stained cultures with either K8 or the antibody for the large T antigen, onto an image analyser and then programming it to quantify the percentage of staining. This method would provide a more accurate measurement of the clonal expansion induced by tumour promoters.

6.2.2 The effect of anti-promoters

Anti-promoting activity was assessed by exposing co-cultures simultaneously to TPA and either retinoic acid and/or fluocinolone acetonide. This study reports no reduction in the clonal expansion of HK-4 cells in co-cultures exposed to TPA compared with those exposed to TPA in the presence of anti-promoters.

Hennings reported anti-promoting activity in the model of initiated mouse epidermis, as assessed by the reduction in the number of colonies of 308 cells in co-cultures following simultaneous exposure to TPA or mezerein and anti-promoters compared with the promoters alone. When these were reassessed by measuring the change in size of the colonies, from photographs in the publication, no significant reduction in clonal expansion was seen in co-cultures exposed to TPA and retinoic acid, or TPA and fluocinolone acetonide, compared with TPA alone. This suggests that neither the human or the mouse model of initiated epidermis identified the anti-promoters that were tested. This could be attributed to molecular differences that have been reported in keratinocytes

in monolayer compared with those in normal skin or within a skin equivalent or due to the timing of anti-promoter application. This has been discussed in more detail in chapters 4. It must be emphasised that neither method of quantifying clonal expansion (either colony size or number) is accurate or reproducible and therefore again quantification of results needs to be addressed.

Overall, anti-promoting activity warrants further investigation. Firstly, more anti-promoters should be tested in this submerged co-culture assay before rejecting the model. The effects of different types of anti-promoter e.g. cyclooxygenase and lipoxygenase inhibitors and free radical scavengers should be assessed in a modified method e.g. anti-promoters should be added at least 24 hours prior to promoters and clonal expansion determined using the computer assisted technique described above.

6.3 Normal skin equivalent

The skin equivalents made up of contracted collagen-fibroblast lattices and seeded with dissociated NHK from the outer root sheath of hair follicles or by directly implanting hair follicle implants are both able to produce a stratified epithelium. The basal cell layer may be identified histologically by the presence of nucleated cells and cornified layers by the presence of non-nucleated squames. Both types of skin equivalent are able to synthesise all keratins found in normal skin. In addition K6 and K16 are present indicating the skin equivalents hyperproliferative nature.

In this study, the skin equivalents produced from hair follicle implants were not as

reproducible as those produced by seeding cultured keratinocytes. Therefore skin equivalents in subsequent studies were synthesised by seeding contracted dermal equivalents with passage 3 keratinocytes derived from the outer root sheath of human scalp hair follicles.

The skin equivalent has not yet been characterised sufficiently. The presence of various markers of differentiation such as involucrin in the spinous layer, filaggrin, epidermal transglutaminase and keratohyalin granules in the granular layer and acylceramides and lanosterol in cornified layers, would give an indication of the presence of each of the layers in the epidermis and also provide information on the extent of differentiation. Histological sections of the skin indicate poor adhesion between dermal and epidermal components, possibly due to a poorly formed basement membrane. It has been suggested that adhesion may be improved by applying a layer of collagen, types III and IV, which are major components of the basement membrane. The poor connection may also be due to low levels of integrin $\alpha_6\beta_4$ and/or bullous pemphigoid antigen which may be investigated using electron microscopy or indirect immunohistochemical techniques.

6.4 A model of initiated human epidermis: co-culture on a skin equivalent

The initiated skin equivalent is at a very early stage of development. Unlike co-cultures in monolayer, keratins cannot be used to distinguish between the normal and premalignant HK-4 keratinocytes. However, an antibody for the large T antigen (416), has been successful at distinguishing between subgenomic SV40 transformed cells from normal keratinocytes when grown at the air-liquid interface. The suitability of this

method in identifying small colonies of HK-4 cells that should form following exposure to a tumour promoter has yet to be investigated. If sensitive enough to identify colonies of HK-4 cells, this model should identify both water soluble and insoluble compounds either by a topical application or dissolved in the medium analogous to systemic exposure. Although this model would require more time to conduct and histological expertise in analysis of results than the co-culture in submerged monolayer, it is still far cheaper and less time consuming than *in vivo* rodent assays. It is therefore important to continue studies investigating its potential for correctly identifying promoters and anti-promoters.

6.5 Conclusions

In vitro models should equal if not exceed animal models in availability, reproducibility and cost effectiveness for common application. The fact that both models described utilise human cells to predict promoting activity in humans cost effectively, in a relatively short period of time suggests that either model has the potential to be used successfully. Continued development and characterisation of the models will determine their applicability in identifying tumour promoters and anti-promoters in industry to help meet the demands placed by the government to evaluate the safety of every new compound synthesised. If successful such models may help to reduce the number of *in vivo* studies that need to be carried out and also the huge backlog of untested compounds already in use.

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APPENDIX I

MATERIALS	SUPPLIER
Cells	
Caco-2	ECACC
Fibroblasts 3T3	ECACC
Fibroblasts GM10	ECACC
HK cell lines	M O'Hare ICR London
Cell Media	
DMEM	Gibco BRL
EMEM	Gibco BRL
Hams F12	Gibco BRL
KSFM	Gibco BRL
Cell Media Suppliments	
Adenine	Sigma
Amphotericin	Gibco BRL
Choleratoxin	Sigma
Epidermal growth factor	Sigma
Foetal calf serum	Gibco BRL
Gentamicin	Sigma
Glutamine	Gibco BRL
Hepes	Sigma
Hydrocortisone	Sigma
Insulin	Sigma
Non essential amino acids	Gibco BRL
Sodium Bicarbonate	Gibco BRL
Sodium Hydroxide	BDH
Sodium Pyruvate	Sigma
Trypsin EDTA	Gibco BRL
Chemicals	
A23187	Sigma
Benzoyl peroxide	Sigma
Dimethyl sulphoxide	BDH
Ethanol	BDH
Fluocinilone acetonide	Sigma
Methanol	BDH
Mezerein	Professor Evans School of Pharmacy
Mitomycin C	Sigma
Sapinotoxin A	Professor Evans School of Pharmacy
Retinoic Acid	Sigma
TPA	Sigma

Immunohistochemistry

Primary antibodies	AE2	ICN
	AE3	Sigma
	K8.6	Sigma
	K8.12	Sigma
	M20	Sigma
	416	Professor Lane, University of Dundee
FITC conjugate		Sigma
Peroxidase conjugate		Sigma
DABfast tablets		Sigma
Glycine		Sigma
Hydromount		BDH

SDS PAGE

Antipain	Sigma
Bis acrylamide	Sigma
Bromophenol blue	Sigma
EDTA	BDH
EGTA	BDH
Glycerol	Sigma
Goat serum	Sigma
Leupeptin	Sigma
2 Mercaptoethanol	Sigma
Pepstatin	Sigma
PMSF	Sigma
SDS	BDH
Sodium azide	BDH
TEMED	Sigma
Tris	BDH

Equipment

Plastic ware for cell culture was bought from Greiner

SDS page was carried out using Mighty Small SE245 Hoeffer

APPENDIX II
CELL MEDIA

All media were prepared under sterile conditions.

Dulbeccos Modification of Eagles Medium (DMEM+)

Basic DMEM+ (with glutamax) supplimented with 10% foetal calf serum (FCS) and 50 ug/ml gentamicin was used to grow 3T3 fibroblasts.

500 ml DMEM

50 ml FCS

2.5 ml Gentamicin (10 ng/ml)

500ml Rheinwalds Medium

Keratinocytes were grown in a modification of Rhienwalds medium:

Rheinwalds medium was made up of a 3:1 mix of DMEM+ with Hams F12 supplimented with 5% foetal calf serum (FCS), $1.8x10^{-4}$ adenine, $1x10^{-10}$ choleratoxin, 10 ng/ml epidermal growth factor, 50 ug/ml gentamicin, 0. 4 ug/ml hydrocortisone, 5 ug/ml insulin.

330 ml DMEM

110 ml Hams F12

25 ml FCS

2 ml gentamicin (10 ng/ml)

1 ml hydrocortisone (0.2 mg/ml) * (5 mg/1 ml 100%ethanol then further dissolved in PBS to make 0.2 mg/ml)

1 ml EGF(5 ug/ml distilled water)*

500 ul choleratoxin(100 nM distilled water)*

500 ul insulin (5 mg/ml 0.1 M HCl)*

1 ml adenine (16.5 mg/ml 0.05 M HCl)*

* These compounds were stored at the concentrations bracketed and aliquots were stored

at -4°C for upto one year except insulin which remains stable for two weeks at 0°C. Aliqouts were sterile filtered with 2 um pore Whatman filters before adding to medium.

NHK were grown in Rheinwalds medium containing choleratoxin RM+C, whilst HK-4 cells were grown in Rheinwalds medium without choleratoxin (RM-C). Choleratoxin is a mitogen in NHK but inhibits the growth of HK-4 cells.

Eagles (EMEM)

EMEM supplimented with 10% v/v FCS, 50 ug/ml gentamicin, 2 mM glutamine, 1% v/v non essential amino acids, 0.2% v/v sodium bicarbonate.

To 375 ml sterilised water add:

EMEM (10X Conc.)	50.0 ml
NEAA (100X Conc.)	5.0 ml
Foetal Calf Serum (10% v/v)	50.0 ml
$NaHCO_3$ (7.5% w/v)	13.0 ml
Glutamine (200 mM)	5.0 ml
Gentamicin (10 ng/ml)	2.5 ml

300 ml 2X EMEM

To 200 ml sterilised water add:

EMEM (10X Conc)	60.0 ml
Amphotericin (250 ug/ml)	6.0 ml
NEAA (100x Conc.)	6.0 ml
$NaHCO_3$ (7.5% w/v)	16.5 ml
Glutamine (200 mM)	6.0 ml
Gentamicin (10 ng/ml)	3.0 ml

100 ml Hair follicle medium

EMEM (section 2.1.5.4) was supplimented with 20 mM Hepes, 1 mM sodium pyruvate and 2.5 ug/ml amphotericin.

EMEM (section 2.1.5.4)	96.0 ml
Hepes (1M)	2.0 ml
Sodium pyruvate (100 mM)	1.0 ml
Amphotericin (250 ug/ml)	1.0 ml

250 ml Skin Equivalent Medium

EMEM was supplimented with 1 mM sodium pyruvate, 10 ng/ml EGF, 0.4 ug/ml hydrocortisone and 10^{-10} M choleratoxin.

EMEM (section 2.1.5.4)	243.0 ml
Sodium pyruvate (100 mM)	2.5 ml

To 1.75 ml EMEM add:

EGF (5 ug/ml)	0.5 ml
Hydrocortisone (0.2 mg/ml)	0.5 ml
Choleratoxin 100 nM	0.5 ml

Sterile filter and add to EMEM and sodium pyruvate.

APPENDIX III

Buffer 1

25 mM Tris- HCl pH 7.4

1 mM EDTA

1 mM EGTA

1 mM phenyl methyl sulphonyl flouride

10 ug/ml antipain

5 ug/ml pepstatin

5 ug/ml leupeptin

Buffer 2

25 mM Tris- HCl pH 7.4

1% SDS

5% Mercaptoethanol

Buffer 3

25 mM Tris- HCl pH 7.4

9M Urea

1% SDS

2% Mercaptoethanol

Running buffer

1.5 mM Tris-HCl. pH 8.8

0.4% w/v SDS

Stacking buffer

0.5 M Tris-HCl pH 6.8

0.4% w/v SDS

Electrode Buffer

25 mM Tris-HCl pH 8.3

192 mM glycine

0.1% w/v SDS

Gel storage solution

100 ml distilled water

3 ml glycerol

2 mg sodium azide

Laemmli buffer (Solubilisation solution 2X strength)

5% SDS, 10% Glycerol, 2% v/v mercaptoethanol, 0.0625 M Tris HCl at pH 6.8.

To make up 10 ml:

1 g SDS

2 ml Glycerol

2 ml Bromophenol blue (0.1% w/v solution in distilled water)

1.25 ml 1 M Tris-HCl pH 6.8

2 ml 2-mercaptoethanol

and make up to 10 ml with distilled water

APPENDIX IV

(a) Human skin
(b) Skin equivalent (cell suspension)
(c) Skin equivalent (hair follicle implants)
(d) HK-4 cells in submerged culture
(e) HK-4 cells grwn at the air-liquid interface

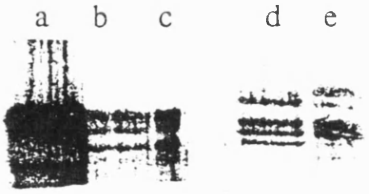

Figure 3.6 page 121
Clonal expansion studies
Effect of TPA on the number of colonies of HK-4 cells in the presence and absence of 3T3 cells

	Mean	SD	SE	n
HK-2				
0.1% DMSO	114.25	3.304	1.652	4
5 nM TPA**	22.8	6.611	2.957	5
20 nM TPA**	22.4	8.433	3.771	5
HK-2 + 3T3				
0.1% DMSO	98.2	14.856	6.644	5
5 nM TPA**	52.0	16.643	9.609	3
20 nM TPA**	40.25	14.220	7.110	4
HK-3				
0.1% DMSO	95.25	14.220	7.11	4
5 nM TPA**	10.8	4.970	2.223	5
20 nM TPA**	11.0	8.083	4.042	4
HK-3 + 3T3				
0.1% DMSO	81.5	6.557	3.279	4
5 nM TPA p>0.05	79.8	23.070	10.317	5
20 nM TPA p>0.05	82.67	9.609	5.548	3
HK-4				
0.1% DMSO	116.0	12.78	6.39	4
5 nM TPA **	36.5	11.733	5.867	4
20 nM TPA **	25.75	5.852	2.926	4
HK-4+ 3T3				
0.1% DMSO	138.8	11.584	5.180	5
5 nM TPA**	72.6	11.415	5.105	5
20 nM TPA**	66.5	14.0	7.000	4

2500 HK -cells were seeded in the absence of 3T3 cells and 500 in the presence of 3T3 cells.

Figure 4.6 page 149
Mean diameter of largest 5 colonies of HK-4 cells following 14 day exposure to TPA , Mezerein or Sapintoxin A

	Mean	SD	SE	n
TPA				
0.1 nM	0.87	0.503	0.291	3
1.0 nM	1.20	0.3	0.173	3
10.0 nM	2.46	0.915	0.409	5
100.0 nM	2.17	0.603	0.348	3
Mezerein				
0.1 nM	0.43	0.126	0.0726	4
1.0 nM	0.87	0.35	0.203	4
10.0 nM	1.73	0.26	0.063	5
100.0 nM	2.35	0.238	0.119	4
Sapintoxin A				
0.1 nM	0.56	0.029	0.017	3
1.0 nM	0.66	0.06	0.033	3
10.0 nM	0.87	0.306	0.176	3
100.0 nM	1.02	0.08	0.04	3
DMSO				
0.01%	0.00			5+
0.1%	0.00			5+

Figure 4.8, page 151
Effect of the anti-promoters retinoic acid and fluocinilone acetonide on Mezerein induced colonies

	Mean	SD	SE	n	
Mezerein 10 nM	1.83	0.173	0.10	3	
Mezerein 10 nM + RA 100 ug/ml	1.90	0.173	0.10	3	p> 0.05
Mezerein 10 nM + FA 10ug/ml	1.86	0.153	0.09	3	p> 0.05

Figure 4.8, page 151
Effect of anti-promoters Retinoic acid and fluocinolone acetonide on TPA induced colonies.

	Mean	SD	SE	n	
TPA 10 nM	2.50	0.26	0.153	3	
TPA 10 nM + RA 10 ug/ml	2.40	0.25	0.115	3	p >0.05
TPA 10 nM + RA 100 ug/ml	2.36	0.152	0.088	3	p>0.05
TPA 10 nM + FA 1 ug/ml	2.17	0.25	0.15	3	p> 0.05
TPA 10nM + RA 10 ug/ml + FA 1 ug/ml	2.32	0.173	0.10	3	p>0.05

Figure 4.7, page 149
Mean diameter of the 5 largest colonies of HK-4 cells, following exposure to the promoters A23187 and benzoyl peroxide.

	Mean	SD	SE	n
A23187 10 uM	0.283	0.029	0.017	3
Benzoyl peroxide 0.1 mM	0.833	0.0.025	0.145	3