Regulation of keratin and integrin gene expression in cancer and drug resistance

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Introduction

Epithelial cells are subject to significant mechanical stress and therefore require a structure to protect them from permanent damage. This structure is the cytoskeleton and it is formed by three classes of proteins: microfilaments (principally actin), microtubules (principally tubulin), and intermediate filaments (IFs) (Steinert and Roop, 1988). The IF proteins differ from the actin and tubulin proteins in several important ways. The IFs are a highly diverse family of proteins. In contrast, there are only approximately 6 forms of actin and six forms each of the 2 types of tubulin (α and β). IF proteins are non-polar, elongated molecules. In contrast, the actin and tubulin proteins are polar and globular molecules. The sizes of the cytoskeletal proteins range from 6 nm (actin) and 10 nm (IFs) to 23 nm (tubulin).

IFs are the most diverse and least understood class of the three cytoskeletal protein families (Steinert and Roop, 1988; Francy *et al.*, 1993). Six classes of IF proteins have been identified: (a) type I acidic keratins, (b) type II basic keratins, (c) vimentin, desmin, glial fibrillary acidic protein, peripherin, and plastin, (d) neurofilaments NF-L, -M, -H and α -internexin, (e) lamins A, B and C and, (f) nestin. At the cell surface the IF proteins are attached to cytoplasmic plaques that are associated with desmosomes and hemidesmosomes. Desmosomes are specialised, cadherin-mediated cell-cell junctions that enable the IFs to form a continuous, multicellular fibrous network. Hemidesmosomes are integrin-mediated adhesive junctions that anchor epithelial cells to the basement membrane.

IF proteins share a number of characteristics. First, they are expressed in both a tissue-specific

and developmentally-regulated manner (except for vimentin). Second, IF proteins have the same overall structure. They contain a conserved central α -helical 'rod' domain and amino- and carboxy-terminal end domains of greatly differing sizes and sequences. The α -helical rod domain contains 310 residues (lamins contain 356 residues) that are highly conserved in charge and secondary structure and to a greater or lesser extent in sequence. The differences between the various IFs arise principally in the amino- and carboxy-terminal domains. Third, analysis of sequence data indicates that the nuclear lamin proteins represent the ancestral precursor for all IF proteins (Dodemont *et al.*, 1990; Döring and Stick, 1990).

This review concentrates on the most characteristic keratins of simple epithelia, namely, K8 and K18. We will give an overview of the current research to date on the regulation of these genes and the non-cytoskeletal functions with which they have been linked.

Keratins

Keratins have a number of characteristic properties (Moll *et al.*, 1982). First, they constitute the largest (>20) family of IF proteins. Second, they are usually expressed only in epithelial cells (Fuchs, 1988). Third, they can be divided into two classes – type I keratins (K9–K20) are smaller and acidic (pKi = 4.5–5.5); type II keratins (K1–K8) are larger and more basic (pKi = 5.5–7.5). Fourth, keratins form obligate heteropolymers, i.e. they form filaments consisting of a pair of keratin proteins, one from each class in a 1:1 ratio. Fifth, keratins are expressed in a tissue-, differentiation-, and developmentally-specific manner in most epithelial cells.

There are two types of epithelia: simple and stratified. As the name implies, simple epithelia are composed of a single layer of cells and they line organs such as the stomach, liver, kidney, etc. Stratified epithelia (such as the epidermis) are composed of several layers with each successive layer representing a more differentiated state. Cells progress from the basal to the upper layers increasing in differentiation as they proceed through the different layers.

Simple epithelia are characterised by the expression of K8 and K18. Some simple epithelia also express K7, K17 and K19 while others express vimentin. Depending on the epithelial cell type each successive layer in stratified epithelia (which are at different levels of differentiation) are characterised by the expression of unique keratin pairs, e.g. the basal layer of stratified squamous epithelia expresses the K5/K14 pair (Fuchs, 1988). The specificity of keratin expression has been used for example in diagnostic clinical determination of carcinomas (epithelial cancers).

Regulation of Expression of the Simple Keratin Genes K18 and K8

The keratins K8 and K18 are the first intermediate filaments expressed in early embryonic development (Jackson *et al.*, 1980). The K8 and K18 genes are unique among the keratin genes for several reasons. First, analysis of amino acid sequence data indicates that they are the most divergent of the keratins suggesting that they represent the ancestral precursors of the other keratins (Blumenberg, 1988). Second, the coding K18 gene is the only human type I gene present in the type II gene cluster on chromosome 12 (Waseem *et al.*, 1990). The other human type I genes are located on chromosome 17 (Ceratto *et al.*, 1997). The relatively close locations of the K8 and K18 genes might have important regulatory implications.

Transcriptional Regulation of K18/mK18 Expression

Of the five kinds of simple epithelial keratins (K8, K18, K7, K17, and K19) the most thoroughly studied at the DNA level is K18 (Oshima *et al.*, 1996). This work has been carried out principally by Robert Oshima and colleagues in The Burnham Institute (formerly the La Jolla Cancer Research Foundation). The human K18 and mouse endoB (mK18) genes were

identified and sequenced in the 1980s (Singer *et al.*, 1986; Kulesh and Oshima, 1988; Oshima *et al.*, 1988; Kulesh and Oshima, 1989). The coding sequences of the genes are highly conserved and the human and mouse genomes contain only one functional gene (Abe and Oshima, 1990). The human genome contains approximately 20 pseudogenes (Kulesh and Oshima, 1988; Oshima *et al.*, 1988) while the mouse genome contains only 3 (Ichinose *et al.*, 1988; Oshima *et al.*, 1988).

The papers published by Oshima's group over the succeeding years have highlighted the complexity of K18 transcription and the roles played by several different factors and elements in regulating this process. Thus far, the most important factors that have been identified are: an enhancer located in the first intron; three negative regulatory elements (NREs) also located in the first intron; a regulatory element in the sixth exon; methylation of an ETS binding site within the first intron enhancer; and an Alu repeat situated in the 5' UTR. A varying combination of these factors controls the transcription of the K18 gene in epithelial (both undifferentiated and differentiated) and non-epithelial cells. The different combinations of factors used depends on the type and the level of differentiation of the cell in question.

Enhancer in the first intron

It was reported in 1990 that a 10 kb restriction fragment (containing the exon and intron sequences, 2.5 kb of 5' UTR and 3.5 kb of 3' UTR) was sufficient for correct tissue-specific expression of the K18 gene in transgenic mice. However, the 5' UTR and/or 3' UTR were not sufficient to drive transcription of a reporter gene (Abe and Oshima, 1990). The intron and/or exon sequences were clearly important for expression. Comparison of the mouse and human K18 sequences identified a 47 bp sequence (located in the first intron) which was conserved between the two species. This sequence plays a key role in transcriptional regulation of the K18 gene. The sequence contained an enhancer which could be activated (via a conserved AP-1 site) in F9 embryonal carcinoma cells (which have very low AP-1 activity) by cotransfection with vectors expressing c-Jun and c-Fos (Oshima *et al.*, 1990). DNase I analysis of the human K18 gene in transgenic mice identified seven tissue-specific hypersensitive sites. Three were located in the 5' UTR, three in the first intron and one in the sixth exon (Neznanov and Oshima, 1993).

Table 1. The classes of intermediate filaments

Class	IF protein	Cell type	Molecular weight (Kd)
I	Type I, acidic keratins	Epithelia	52.5–68
II	Type II, basic keratins	Epithelia	40.0–64
III	Vimentin	Mesenchyma	54
	Desmin	Muscle	52
	GFAP	Glial cells; Astrocytes	53
	Peripherin	Peripheral nervous system	57
	Plasticin	Retinal ganglion cells	52.6
IV	Neurofilament proteins -L, -M, -H, α -internexin	Central nervous system	68, 145, 200
		CNS neuronal cells	66
V	Lamins A, B, C	Nuclear membrane	60, 67, 70
VI	Nestin	Neuroepithelial cells	200

Further studies by Oshima's group led to the identification of a second transcription factor binding site within the conserved 47 bp of the first intron. This site is recognised by members of the ETS transcription factor family (Pankov *et al.*, 1994a).

The AP-1 and ETS sites regulate to a large extent the expression of the K18 gene in transgenic mice. There is a close functional relationship between the two binding sites. Mutation of either site greatly reduces expression of the K18 gene in transgenic mice while mutation of both reduces expression by up to 85% (Oshima *et al.*, 1990).

Mouse F9 embryonal carcinoma cells do not synthesise keratins K8 and K18 until induced to differentiate. This lack of expression is due in part to the low levels of the ETS and AP-1 transcription factors. After induction of differentiation the levels of ETS-2, c-Jun and JunB rise and expression of the endogenous mK18 and transfected human K18 genes ensues (Pankov *et al.*, 1994a). Activation of the K18 gene in vivo occurs via the first intron enhancer through oncogenic activation of the Ras signaling pathway (Pankov *et al.*, 1994b). Therefore, the AP-1 and ETS sites together constitute an 'oncogene-responsive regulatory element'. However, the conserved enhancer element, while necessary for transcriptional activation, is not sufficient by itself (Oshima *et al.*, 1990). Other factors are clearly important.

Negative regulatory elements in the first intron

Three negative regulatory elements (NREs) located in the first intron of the K18 gene also play a part in transcriptional regulation of the K18 gene in undifferentiated embryonic cells (Pankov *et al.*, 1994a). Two

of the NREs are conserved in mK18. The NREs are functional in undifferentiated F9 cells but not in somatic cells. The levels of the proteins which bind to these elements decrease upon differentiation. In addition, deletion of the NREs results in increased activity of the first intron enhancer (Pankov *et al.*, 1994a). Binding of proteins to the NREs is not evident in somatic cells. This indicated that in these cells permanent repression of K18 transcription is not due to the effect of the NREs.

Regulatory element in exon 6

Several pieces of evidence have indicated that exon 6 is important in transcriptional regulation of K18 among which is (a) a DNase I hypersensitive site is located in the exon and, (b) deletion of parts of exons 6 and 7 reduces transcription in transfected cells which can be restored upon reintroduction of exon 6 (Neznanov and Oshima, 1993).

Neznanov *et al.* (1997) have shown that an element within exon 6 containing an AP-1 site partly regulates K18 expression. This element stimulates the proximal promoter and interacts with the first intron enhancer to control the regulation of the gene by AP-1. This response is dependent upon the presence of the first intron enhancer. Regulation by exon 6 probably also involves other binding sites as (a) deletion of the AP-1 site decreases but does not abolish completely the K18 transcription and (b) the exon can regulate transcription independently of AP-1.

Methylation of ETS site

Methylation of the CpG islands located throughout the human genome has been implicated in the regulation of expression of a wide number of genes. This is also the case for K18. 5-azacytidine (an inhibitor of DNA methylation) induces expression of mK18, mK8 and mK19 in a myoblast cell line (Darmon, 1985). Methylation accounts for the lack of correlation between expression of the oncogenes c-Jun, c-Fos, etc., and K18 expression in epithelial cells. The methylated sequence is the ETS site in the first intron. Methylation of this site shows an inverse relationship to the expression of K18 in a number of tissues (Umezawa *et al.*, 1997). The CpG-rich regions of the first exon appear to be unmethylated in transgenic mice (Neznanov and Oshima, 1993).

Alu repeat in 5' flanking region

The 5' flanking region of the K18 gene contains two copies of an Alu repeat element. Alu repeats are short repetitive DNA sequences which are found throughout primate genomes. They have an average length of 282 bp, are present as 500 000–1 000 000 copies in the haploid human genome and comprise approximately 5% of the human genome (Shen *et al.*, 1991). The repeat closest to the gene (proximal) contains two of the previously identified DNase I hypersensitive sites (Neznanov and Oshima, 1993). This Alu element is also conserved in position and orientation in mK18 which would indicate that it might have a functional role. In most transgenic experiments the introduced genes are expressed in an integration site-dependent and copy number-independent manner. Unusually, the transfected K18 genes in transgenic mice are expressed in an integration site-independent and copy number-dependent manner (Thorey *et al.*, 1993). This indicates that there is an element in the introduced K18 sequences which transcriptionally insulates the K18 gene.

Thorey *et al.* (1993) reported that this element was the proximal Alu repeat. The repeat is in the opposite orientation to the K18 gene and produces a transcript in transgenic mice. The level of this transcript correlated well with expression of the K18 gene which indicated that it in some way regulated expression of the K18 gene in transgenic mice. The integration site-independence property of the K18 transgene requires the presence of either the 3' UTR or a 825 bp fragment of the 5' UTR containing the Alu element. Mutation of the RNA polymerase III promoter of the Alu repeat

abolishes copy number-dependent expression but does not affect the integration site-independence property of the transgene in the presence of the 3' UTR. The proximal repeat also contains a functional retinoic acid receptor element (RARE). This is discussed below.

Summary

From the papers published to date it appears that there are several levels to regulation of K18 gene expression. First, in undifferentiated murine epithelial cells transcriptional repression of the K18 genes is achieved by a combination of 3 factors: methylation of the ETS binding site within the enhancer; low levels of the AP-1 and ETS factors; and binding of factors to the negative regulatory elements of the first intron. As the cells undergo differentiation the level of the AP-1 and ETS factors rises, demethylation occurs and the negative factors dissociate from the NREs. Second, in nonpermissive tissues the chromosomal architecture of the surrounding DNA appears to play a role in permanently shutting down the K18 gene.

Transcriptional Regulation of K8/mK8 Expression

The simple keratin K8 is the obligate heteropolymer of K18. Isolation of the human K8 gene and the mouse homologue endoA (mK8) genes (Vasseur *et al.*, 1985; Morita *et al.*, 1988; Krauss and Franke, 1990; Tamai *et al.*, 1991) has permitted the investigation of K8 gene regulation. The research carried out to date suggests that regulation of K8/mK8 expression is complex and that the mechanisms employed depend on the cell in the question. Several different factors have been implicated.

Transcriptional silencing by a labile inhibitor

The first mechanism for regulation of mK8 expression was suggested by Cremisi and Duprey (1989). Undifferentiated murine PCC embryonal cells normally do not express mK8. However, treatment of these cells with cycloheximide or differentiation of the cells, led to expression of the mK8 gene. Moreover, treatment of 3T6 fibroblast cells (which do not express mK8) with cycloheximide had no effect. This suggested that the method of transcriptional silencing differed in the PCC and 3T6 cells. These results lead to the conclusion that in undifferentiated PCC cells transcription of the mK8 gene is repressed (at least partially) by a

labile inhibitor and that expression of the gene is controlled by positive and negative factors (Cremisi and Duprey, 1989). This is similar to the regulation of K18 in undifferentiated murine EC cells.

It is worth considering the reports investigating the expression of K18/mK18 when considering which other factors may be involved in mK8 expression. A report by Onclercq *et al.* (1989) suggested the possibility that AP-1 may be involved in mK8 transcription. Oshima's group has shown that AP-1 (together with ETS) regulates human K18 expression through an enhancer in the first intron and an AP-1 site in exon 6. There are three possible AP-1 binding sites in mK8. These are located in the first exon, first intron and 3' UTR. Since a member of the ETS family binds to the 3' UTR of mK8 it will be fascinating to see if a role for AP-1 in mK8 expression is also discovered. Significantly, DNase I analysis of mK8 found a number of hypersensitive sites in the 3' UTR (enhancer), first exon and first intron (Miyashita *et al.*, 1993).

Enhancer in the 3' UTR of mK8

Recent reports by Nozaki and colleagues in Osaka University have detailed the positive transcriptional role played by the 3' UTR of mK8. They have demonstrated that the mK8 promoter is activated by a 3' enhancer (Takemoto *et al.*, 1991). Following this it was reported that the enhancer is located 1 kb downstream of the termination codon and consists of six direct repeats of 22 bp, with two ETS binding sites (termed EBS1 and EBS2) with GGAA as a core (Fujimura *et al.*, 1994; Seth *et al.*, 1994). The EBS1 site is essential for the activity of the enhancer while EBS2 adds to the enhancing effect. The EBS1 site binds a protein with the same binding specificity as Ets-2, but is not Ets-2. The enhancing effect is tissue-specific in that it drives expression in endodermal cells. It appears likely that there are other positive, cell-specific factors involved in this process as the enhancer effect varies between cell types (Takemoto *et al.*, 1991).

Comparison of the 3' UTR sequences of the K8 and mK8 genes fails to identify similar ETS sequences in the K8 gene. In addition, work carried out by Casanova *et al.* (1995) has shown that the 5' and 3' UTRs of the K8 gene are not sufficient for expression and that elements within the body of the K8 gene were crucial for expression. This means that either (a) the K8 3' UTR does not contain an enhancer or (b) it is active only in early development.

Interestingly, the human K8 genes in transgenic mice exhibit the same integration-independent and copy number-dependent expression as K18 genes (Casanova *et al.*, 1995). We may speculate that this could be due to the presence of similar Alu repeats in the 5' UTR and first intron of K8 as the proximal Alu element in the 5' UTR of K18 which confers transcriptional insulation upon this gene in transgenic mice (Thorey *et al.*, 1993). It would be interesting to discover if either of the K8 Alu repeats produces a transcript as does the proximal Alu repeat of K18.

Methylation

It is probable that methylation plays a role in repressing transcription of the mK8 gene as treatment of teratocarcinoma-derived fibroblasts by 5-azacytidine (an inhibitor of DNA methylation) results in mK8 expression (Darmon, 1985). Similarly, the CpG region of the mK8 gene around the first exon was found to be hypomethylated in endodermal PYS-2 cells and F9 embryonal cells and hypermethylated in 3T6 fibroblasts which do not express mK8 (Tamai *et al.*, 1991).

cis-acting negative regulatory elements

A sequence comparison that we have carried out between the K8 and K18 sequences has identified two sequences in K8 which are similar to the three NREs in K18. These are located at -779 in the 5' UTR and +6442 in the fifth intron. Because a labile inhibitor has been implicated in the regulation of mK8 expression it is interesting that such similar sequences should also be present in the human K8 gene.

Keratins and p53

Interestingly, and perhaps surprisingly, the tumour suppressor gene p53 has been linked to the regulation of keratin transcription by a small number of recent papers. The first report indirectly linking these genes was by Ledinko and Costantino in 1990. They reported the retinoid-mediated downregulation of p53 and K18 and decreased invasiveness in the human lung cancer cell line A549. Mukhopadhyay and Roth showed that wild-type p53 transfected into the p53-negative human lung cancer cell line H358 regulates expression of K8 (Mukhopadhyay and Roth, 1996a). They inactivated p53 by antisense RNA which downregulated K8 synthesis and increased the invasive ability of lung cancer carcinoma cells (Mukhopadhyay and

Roth, 1996b). The association between p53 and K8 is exciting considering the body of work that has indicated a role for K8 and K18 in increasing tumour invasiveness. The work of Ledinko and Costantino and of Mukhopadhyay and Roth suggests an inverse relationship between keratin expression and invasiveness in p53-negative lung cancer cells. This would agree with a previous suggestion that increased invasiveness (associated with keratin synthesis) is evident in cell lines which would not normally express the keratins. It remains to be seen how important a role p53 plays in regulating K8 transcription *in vivo*.

Summary

The lack of expression in undifferentiated epithelial cells has been accounted for by the presence/absence of negative and positive factors (Cremisi and Duprey, 1989). It is envisaged that in undifferentiated F9 or PCC cells a surplus of negative factor(s) and a lack of positive factor(s) results in transcriptional repression. Upon induction of differentiation this imbalance is reversed and expression of the K8 gene ensues. Therefore, these results support the following model for the regulation of mK8. In nonepithelial cells (which do not normally express mK8) transcription of the gene is irretrievably shut down by methylation of the CpG islands. In undifferentiated epithelial (which upon differentiation will express mK8) the CpG islands are hypomethylated and expression is controlled by a balance between positive and negative factors.

Posttranscriptional Regulation of K8 and K18 Expression

The K8 and K18 proteins are regulated at the transcriptional level in most epithelial and non-epithelial cells. However, there are several examples where control is exercised posttranscriptionally at the translational and posttranslational levels. In addition to K8 and K18 posttranscriptional regulation has also been demonstrated for K6 and K16 (Tyner and Fuchs, 1986), and for K5 and K14 (Paine *et al.*, 1992).

Posttranslational regulation has been documented by several groups. In the absence of its partner both K8 and K18 proteins are rapidly degraded by a proteolytic system in both simple epithelial cells and SV-40-transformed fibroblasts (Knapp and Franke, 1989; Kulesh *et al.*, 1989).

In a study of K7, K8 and K18 expression in normal, dysplastic and malignant oral epithelia Su and

colleagues (1994) have shown that K8 and K18 are regulated posttranscriptionally. In normal epithelia the K8 and K18 mRNA (but not the corresponding proteins) were present in basal and spinous cells but not the proteins. The mRNAs were translated in approximately half the dysplastic cells and all the malignant carcinomas. The authors suggest that this posttranscriptional regulation is due to the release of the epithelial cells from a block on mRNA translation or rapid degradation of K8 and K18 protein in normal epithelia. This association between expression of K8 and K18 and malignancy is interesting in the light of other studies which have implicated these keratins in increasing tumorigenicity (see below).

Blouin *et al.* (1992) reported a different form of posttranscriptional regulation of K8, K18 and K14 expression in T51B (a rat liver nonparenchymal cell compartment cell line) which expresses K8 and K14 (K8 pairs usually with K18 and K5 pairs with K14, in the absence of their traditional partners K8 and K14 will form intermediate filaments). Subclones developed through exposure to nickel were found to express K8 and K18 with the loss of or low level K14 expression. The control of K18 expression seemed to be posttranscriptional in that the K18 mRNA levels increased but not due to a corresponding increase in K18 gene transcription or K18 mRNA stability. The observed differences may be due to differences in (a) the degradation or maturation of nascent transcripts or (b) the control of nuclear RNA processing. The data obtained on the mRNA levels suggested that the regulation of IFs in T51B cells differs widely depending on the gene. For example, in these cells, the control of vimentin expression was reported to be transcriptional only, while that of K18 appeared to be primarily posttranscriptional, via changes in nuclear mRNA processing with perhaps an increased efficiency in converting translationally inactive Poly(A) K18 hnRNA to active Poly(A) K18 mRNA. K8, on the other hand, appeared to be controlled at both the transcriptional and the posttranscriptional level. So, it would appear that even within one simple epithelial cell line there are distinct and diverse molecular mechanisms used to control the expression of different keratin pairs.

Bisgaard *et al.* (1994) reported the abrogation of IF expression and induction of K18 and α -fetoprotein upon the spontaneous differentiation of rat liver epithelia, which normally express K18 and vimentin, to hepatoblast-like progeny. Chemically transformed cells expressed K18 and K14, but not K8, while cells spontaneously transformed by prolonged passage in

culture expressed K8 and K18, but not K14 or vimentin. Nuclear run-on assays showed that K14, K18 and vimentin were transcriptionally regulated, while K8 appeared to be posttranslationally regulated. The K8 mRNA was found in all cells, but the K8 protein was only evident in those cells that expressed either K14 or K18. This posttranslational regulation is commonly reported for keratins where an unpaired keratin filament is proteolytically degraded in the absence of a partner with which to form a stable filament structure. Keratin and vimentin filaments seem to be differentially regulated depending upon the agent used for transformation, as in this case where K14 partnered K8 in chemically transformed cells whereas K18 accompanied K8 upon spontaneous transformation. It is likely that different agents affect different control points in the complex regulatory mechanism of keratin expression, and therefore examining the effects of a number of agents on the one keratin in a particular cell line should begin to reveal the various levels of control used by cells for the regulation of expression of these filaments.

Retinoic Acid Alters Keratin Expression

Vitamin A plays a key role in vertebrate development and in maintaining homeostasis by acting as a biological signal to control cell growth and differentiation. Retinoic acid (RA), a metabolic derivative of vitamin A, promotes an undifferentiated phenotype in cultured cells. Intracellular retinoic acid receptors (RARs) bind to specific hormone response elements (HREs) to regulate expression of target genes. Two such targets are the K8 and K18 genes. RA induces expression of keratins which are characteristic of simple epithelia (K8, K18, K7, K17 and K19) while it represses the expression of the other keratins found in stratified epithelia (K1/K10; K5/K14; K6/K16).

RA appears to exert its effects in the cytoplasm through the cellular retinoic acid binding proteins (CRABP) I and II. In the nucleus RA acts through two sets of transcription factors: the retinoic acid receptors RAR α , β and γ and RXR α , β and γ . The RARs belong to a family of transcription factors that also include the thyroid and steroid hormone receptors. The RXR factors are believed to dimerise with the RAR factors, thyroid hormone and vitamin D receptors to exert their influence.

The effects of retinoic acid (RA) on keratin expression depend on the degree of differentiation of the cells

in question, i.e. in some lines expression of the keratin genes will be upregulated while in others expression will be downregulated or remain unaffected. It also appears that the level at which RA exerts its influence, i.e. transcriptionally or posttranscriptionally, can vary depending on the cell line/type. This is partly because the levels of the RARs vary among cell lines.

A significant difference between the induction and repression effects of RA is the lag-time between treatment and effect. Most RA-mediated changes are at the level of mRNA. In the case of induction (simple epithelia) the effect is relatively slow occurring over 48–96 h post RA-treatment. This response is therefore likely to involve several intermediate factors and not just proteins binding to HREs within a keratin gene. The repression effect (stratified epithelia) is much quicker occurring within 6 h. The rapidity of the repression effect suggests that the retinoic acid receptors either interact directly with the RAREs or with the minimum use of intermediate factors.

The 5' UTR of K18 contains two Alu repeats, both of which contain four copies of sequences homologous to a HRE. Together, in the proximal Alu repeat, the HREs constitute a retinoic acid response element (RARE). The HREs consist of hexamer sequences (consensus AGGTCA) arranged as direct repeats separated by 2 nucleotides. Three of the HREs, HRE 23 and HRE 34, can bind RA receptor proteins. The binding sites increase expression of a reporter gene in transiently transfected CV-1 cells by up to 35-fold (Vansant and Reynolds, 1995). Recent results demonstrate that RA-induced K18 expression is dependent on the enhancer in the first intron. RA induction of F9 EC differentiation has shown that K18 induction requires the synthesis of c-jun, junD and/or ETS which bind to the enhancer containing the AP-1 and ETS sites (Pankov *et al.*, 1994a).

Keratins and Intermediate Filament Attachment Proteins (IFAPs)

Much of the research into keratins within the last decade has striven to elucidate their non-structural functions. For example, it has been suggested that IFs can act as signal transducers, relaying information from the extracellular matrix (ECM) to the nucleus (Skalli and Goldman, 1991).

Non-IF proteins which have given hints to the non-structural functions of keratins are the intermediate filament attachment proteins (IFAPs). Several of these

have been identified within the last decade. They include desmoplakin, IFAP300 and plectin (components of both desmosomes and hemidesmosomes). A number of IFAPs have also been linked to the simple keratins K8 and K18. Work by Omary and colleagues has identified hsp70 (Liao *et al.*, 1995), PKC ϵ (Omary *et al.*, 1992) and the family of proteins collectively known as 14-3-3 (Liao and Omary, 1995). The interaction between 14-3-3 and K8/K18 is phosphorylation- and cell cycle-dependent, i.e. it occurs only when the keratins are hyperphosphorylated during the cell cycle. The association occurs preferentially with K18 at a ratio of one or more 14-3-3 molecule/keratin tetramer. Dephosphorylation of the keratin destroys the binding reaction. Interestingly, the known functions and interactions of 14-3-3 (with the signal transduction kinases such as PKC ϵ and the Ras signalling pathway) may provide a clue to a possible role of the keratins in intracellular signalling. Significantly, the 14-3-3 proteins are Raf-I activating proteins (as is Ras-I) which is interesting as K18 transcription is activated via the Ras signal transduction pathway.

Omary's group has also demonstrated binding between hsp70 proteins and K8(preferentially)/K18 (Liao *et al.*, 1995). This interaction is ATP-dependent and does not interfere with the binding between 14-3-3 and K8/K18. A primary role of heat shock proteins is to act as molecular chaperones. They promote the folding of newly synthesised proteins and their assembly into larger structures. Does this suggest that keratins also may play a role in this function.

In conclusion, the very specific interactions between K8/K18 and IFAPs indicate that the former perform a number of roles other than their structural functions. It is clear that the years ahead should provide a wealth of data to elucidate these non-structural functions.

Keratins and Multidrug Resistance

One of the greatest barriers to the successful treatment of cancer is the acquisition of multidrug resistance (MDR) by tumours. This may have several causes either intrinsic or induced (through selection of MDR cells by treatment with chemotherapeutic agents). MDR is usually associated with the expression of proteins such as *mdr-1* and MRP (multidrug resistance-associated protein). However, the greater intrinsic drug resistance of epithelial cells as compared to, for example, hematopoietic cells has prompted the

question of whether this difference might be partly due to a defining characteristic of the former, namely keratins.

Several recent reports have linked IFs and MDR. For example, K8 and vimentin protein levels increase significantly in OAW42 cells exposed to doxorubicin (OAW42-A) as compared to the sensitive parental cells (OAW42-S) (Moran *et al.*, 1997). However, in other cell lines, for example human colon carcinoma cell clones (LoVo) only the vimentin protein shows such an increase (Conforti *et al.*, 1995). Bauman *et al.* (1994) transfected mouse L fibroblast cells with K8 and K18 cDNA. They demonstrated an MDR phenotype and increases in resistance in response to treatment with mitoxantrone (7.6-fold), doxorubicin (5.2), methotrexate (4.9), melphalan (6.5), colcemid (17.2) and vincristine (454). This increase was not due to altered growth characteristics, drug accumulation, or drug efflux. Three other agents (cisplatin, UV and ionising radiation) produced no effect.

The results for cisplatin from this work are in agreement with other studies which have demonstrated an inverse relationship between keratin expression and cisplatin-resistance. In one study expression of the type I keratin K14 was reduced in human lung squamous carcinoma cells exhibiting cisplatin resistance (Katabami *et al.*, 1993). In a second study an inverse link between cisplatin sensitivity and K18 expression was demonstrated in human ovarian adenocarcinoma cells (Parekh and Simpkins, 1995). The cisplatin-resistant cells (low K18 expression) were also hypersensitive to the effects of taxol, vincristine, and vinblastine (Parekh and Simpkins, 1996).

Cress and Dalton (1996) have suggested four pieces of evidence in support of C-MDR (cytokeratin-dependent multidrug resistance). First, there is a clear direct association between keratin expression and MDR in epithelial tumours. Second, expression of K8 and K18 cDNA in keratin-negative cells confers C-MDR (Bauman *et al.*, 1994). Third, selection of keratin positive cells also selects for MDR (Anderson *et al.*, 1996). Fourth, C-MDR was noted with five different chemotherapeutic agents, only two of which have similar modes of action. Interestingly, the same authors have shown that the C-MDR response does not require a complete keratin filament network. A mechanism which might explain this finding has not yet been advanced.

Coexpression of Keratins with Vimentin Increases Tumorigenicity and Invasiveness in Some Cell Lines

It has become increasingly clear in recent years that there is an important correlation between IF protein expression and metastasis (Hendrix *et al.*, 1996). The evidence for this is multifold: (I) Solid epithelial tumours (and their primary carcinomas and metastases) normally express only keratin filaments. Vimentin expression occurs once the tumour cells are shed into ascitic or pleural fluid (Ramaekers *et al.*, 1983). (II) The non-metastatic rat pancreatic BSp73 adenocarcinoma expresses only vimentin while the metastatic counterpart expresses both vimentin and keratins (Ben-Ze'ev *et al.*, 1986). (III) Metastatic human melanoma cells express both vimentin and keratins unlike the non-metastatic state which expresses only vimentin (Hendrix *et al.*, 1992; Chu *et al.*, 1996). (IV) Co-expression of vimentin and keratin in MCF-7 human breast cancer cells increases invasiveness (Hendrix *et al.*, 1997). (V) Transfection of mouse L cells (which express only vimentin) with K8 and K18 cDNA increases cell migration and invasion (Chu *et al.*, 1993).

A recent report suggests that the simple keratins may only increase the motility of those cells which do not normally express keratins (Pankov *et al.*, 1997). In the non-metastatic BSp73 carcinoma it was found that clones expressing K18 alone (or together with K8) were less motile compared to clones expressing a K8/K19 pair. Therefore, it appears that K18 reduces motility of this adenocarcinoma. The K18-expressing cells had a lower tumorigenicity than the K8/K19-expressing cells.

Hendrix and colleagues have postulated that the increased invasive behaviour of epithelial cancer cells expressing both vimentin and keratins may be the result of a selective advantage of the cells in interpreting signals from the extracellular matrix (Hendrix *et al.*, 1996).

Regulation of Integrin Expression

Introduction

Integrins are a large family of heterodimeric transmembrane glycoproteins that bind to components of the extracellular matrix and can bind to cell-cell adhesion molecules such as CAMs of the immunoglobulin family (Newham and Humphries, 1996). Integrins

consist of two protein chains; the α chain is known to have 15 variants while the β chain has 8 identified variants at present. They can combine into at least 22 different integrins. The α subunits are homologous to one another but not to the β subunits, which form their own homologous group. The extent of similarity at the amino acid sequence level within the α and β subunit groups is 40–50%.

Integrins are versatile adhesion receptors expressed by almost every cell type. In addition to mediating cell adhesion, integrins are now known to function as signalling receptors, participating in a diverse array of cellular events including spreading, migration, proliferation, differentiation, apoptosis, and gene expression. Epithelial cells, *in vivo*, perform a number of important functions that require unique interactions with the ECM. These include wound repair, modulation of inflammation and establishment of tissue polarity and morphogenesis. Integrins have been implicated in a number of diseases and in cancer (Hillis and MacLeod, 1996; Horwitz, 1997). Neoplastic change seems to be associated with alterations in cellular integrin expression. Integrins play a role in a number of cellular processes that impact on development of tumours, including the regulation of proliferation and apoptosis, cellular motility and invasion, cell surface localisation of metalloproteinases, and angiogenesis.

Structure of integrins

The α subunits vary in size from 150 to 200 kDa and are non-covalently associated with the β subunit which varies in size from 90 to 110 kDa, with the exception of β_4 which is larger than 210 kDa. The integrin receptor is a transmembrane glycoprotein with two large globular amino terminal extracellular domains that together make up an ellipsoidal head (N-domain). Each subunit provides a relatively thin leg which traverses the plasma membrane and ends in a relatively short cytoplasmic tail of less than 60 amino acids (C domain). The only known integrin which does not fit this category is the β_4 integrin which has a cytoplasmic domain of close to 1000 amino acids (Akiyama *et al.*, 1995).

The β subunits all show considerable similarity at the amino acid level (Hemler, 1990). They all contain 56 conserved cysteine residues except for β_4 which has 48. These cysteine residues are arranged in 4 repeating patterns which are thought to be linked in-

ternally by disulphide bonds. The α subunits exhibit a lower degree of similarity than the β chains. The N-terminal region of all α subunits contain a sevenfold tandem repeat of a homologous module composed of approximately 60 amino acids. The final 3 or 4 of these repeats show sequence homology to functional subunits known as EF-hands, found in Ca^{2+} binding proteins such as calmodulin. Seven α subunits including α_1 and α_2 chains of β_1 integrins plus the α_L , α_M and α_X chains of β_2 integrins possess a 200-residue insert between the second and third repeats which shares sequence homology with the A domain of von Willebrand factor and a number of ECM components. This may contribute to the binding function of these integrins (Hemler, 1990).

Several divalent cations regulate integrin-ligand binding including Ca^{2+} , Mg^{2+} and Mn^{2+} . The activity varies with the integrin. Integrins possess multiple cation binding sites and it is not clear whether occupancy of one or all of these sites is required for activation or ligand binding. A characteristic feature of integrins is that they can exist in active or inactive states where the transition from the inactive to active state appears to be accompanied by conformational changes and is regulated by divalent cation occupancy (Hillis and MacLeod, 1996).

The α and β subunits could in theory associate to give more than 100 integrins. However, the actual diversity is much more restricted. The β_1 integrins primarily affect adhesion between cells and the ECM (Buck and Horwitz, 1987), the exception being α_4 which mediates cell-cell adhesion *via* VCAM-1 (Elices *et al.*, 1990). The majority of β_1 integrins are receptors for ECM proteins such as collagens (α_1 , α_2 , α_3), fibronectin (α_3 , α_4 , α_5) or laminin (α_1 , α_2 , α_3 , α_6). The β_2 integrins are composed of three closely related molecules which are also known as leucocyte integrins because their expression is limited to leucocytes (Springer, 1990). They participate in cell-cell interactions (Hynes, 1987). The β_3 integrins consist of two members at the moment. The $\alpha_{IIb}\beta_3$ integrin is involved in platelet aggregation and has affinity for fibronectin, fibrinogen and von Willebrand factor (Ferrell and Martin, 1989). The $\alpha_v\beta_3$ receptor binds to many adhesion molecules including vitronectin and thrombospondin and appears to have an important role in angiogenesis (Brooks *et al.*, 1994). The binding specificity of a particular integrin may vary according to cell type which further adds to their complexity. $\alpha_2\beta_1$ is a receptor for collagen on platelets but is

a collagen and laminin receptor on endothelial cells (Kirchofer *et al.*, 1990).

Integrins hold a cell in place by attaching at one end of the molecules of the ECM (or molecules on other cells) and at the other end to the cellular cytoskeleton. Binding is not very tight, but many relatively weak attachments form a stronger one. The most extensively studied site in the integrin ligands is the RGD sequence. This sequence is present in fibronectin, fibrinogen, thrombospondin, vitronectin, vWF, laminin and collagen type I (Ruoslahti and Pierschbacher, 1987; Humphries, 1990). Integrin ligands appear to use very short (3 or 4 amino acids) sequences as recognition motifs for receptor binding. An additional binding site in fibronectin and in some cell-surface Ig superfamily ligands has led to the identification of a second common motif 'LDVP' (Humphries, 1990). This binds to $\alpha_4\beta$ integrin. The 'DGEA' site in collagen type I was also recognised (Staatz *et al.*, 1991) among others (Newham and Humphries, 1996). All these sequences have been shown to possess a degree of cross-reactivity in their binding to a variety of integrins. However the exact mechanisms by which integrins bind different ligands are not yet fully understood.

Integrin signalling

Integrins function as signalling receptors to direct cell adhesion and regulate other aspects of cell behaviour, including cell proliferation and differentiation, and to determine cell survival and cell cycle events (including apoptosis). This can mediate the bidirectional transfer of information from the outside to the inside of the cell, and also from the inside to the outside of the cell, which has been termed 'outside-in' and 'inside-out' signalling respectively. The pathways involved in integrin signalling have been extensively reviewed in the last few years (Dedhar and Hannigan, 1996; LaFlamme and Auer, 1996; Ruoslahti, 1997).

Although the cytoplasmic domains of both integrin subunits are required for binding, the β chain appears particularly important. Phosphorylation of this subunit may be a means by which the function of this integrin is regulated (Hibbs *et al.*, 1991). β_1 , β_2 , β_3 , β_4 , β_5 and β_6 chains all possess potential sites for phosphorylation due to the presence of tyrosine residues. Thus, the β subunit is primarily responsible for integrin clustering to focal adhesions (LaFlamme *et al.*, 1992).

Occupation of integrins at focal adhesions results in tyrosine phosphorylation of focal adhesion kinase (FAK) and regulates actin cytoskeleton reorganisation. This is termed 'outside-in signalling'. p21^{ras} is a member of the Ras superfamily of GTPases which regulates actin cytoskeleton rearrangement and assembly of focal adhesion plaques in response to growth factors and ligand binding to the ECM (Ridley and Hall, 1992). Assembly of focal adhesions requires the direct binding of the cytoplasmic domains of the β integrin subunit with the focal adhesion proteins, α -actinin, talin and FAK. FAK can bind to paxillin which is tyrosine phosphorylated in an adhesion-dependent fashion (Parsons, 1996). FAK has binding sites for the SH2 domain of Src and for phosphatidylinositol-3-kinase (Clark and Brugge, 1995).

The phosphorylation of FAK is believed to initiate a cascade of phosphorylation events and new protein interactions required for adhesion and formation of adhesion-dependent signalling complexes. The adhesion-dependent activation of MAP kinases appear to be important in the pathway by which integrins regulate cell proliferation. MAP-K can then be activated by Ras which can in turn activate a number of transcription factors including *c-myc* that can regulate growth and differentiation (Davis, 1993). Another early event triggered by integrins is the activation of lipid secondary messenger pathways. Activation of tyrosine kinase dependent phospholipase C- γ (PLC- γ) has been demonstrated which in turn hydrolyses phosphatidylinositol (PIP₂) to inositol triphosphate and diacylglycerol which are secondary messengers involved in the control of intracellular Ca²⁺ and the activation of protein kinase C (PKC) (Kanner *et al.*, 1993; Daniel *et al.*, 1992). PIP₂ can also regulate other cell processes, such as modulating the function of several actin-binding proteins of the cytoskeleton.

'Inside-out signalling' refers to changes in the activation state of integrins *via* intracellular signalling. The ability of cells to regulate their adhesiveness is critical in many situations. Modulation of the ligand-binding activity of integrins plays a role in these responses and is critically dependent on the cytosolic environment with which the tails of the integrins are associated. There appear to be regions of the cytoplasmic tails which may be crucial for the mediation of this 'inside-out' signalling. A highly conserved 'GFFKR' motif exists in many α subunits (O'Toole *et al.*, 1994; Kassner *et al.*, 1994).

Functional Role of Integrins

Ligation of integrins by their ECM protein ligands induces a cascade of intracellular signals, including an increase in intracellular pH and intracellular Ca²⁺ levels (Schwartz, 1992), tyrosine phosphorylation of pp125 FAK (Kornberg *et al.*, 1993), activation of p34/cdc2 (Symington, 1993) and cyclin A (Guadagno *et al.*, 1993), synthesis of protein kinase C (Vuori and Rouslahti, 1993), activation of MAPK (Chen *et al.*, 1994b; Schlaepfer *et al.*, 1994; Morino *et al.*, 1995), p21 ras (Kapron-Bras *et al.*, 1993), and transcription factors such as NF- κ B (Yebra *et al.*, 1995). As well as signal transduction, integrins have many roles in mediating the expression of genes involved in proliferation, apoptosis, differentiation, invasion and metastasis.

Role of integrins in proliferation

Most adherent cells are incapable of proliferating without signals from the ECM, most of them being transmitted *via* integrins. Integrin signalling has been found to regulate cell-cycle progression. In fibroblasts, the requirements for adhesion maps to the G₀/G₁ transition and to mid-to-late G₁ before the initiation of DNA synthesis (Guadagno *et al.*, 1993). Integrin-mediated signalling activates expression of MAP kinases, which have been implicated in cellular proliferation (Davis, 1993), as discussed previously. Cyclin A expression, which is required for entry into S phase of the cell cycle, is regulated by adhesion (Guadagno *et al.*, 1993).

There may be a possible tumour suppressor role associated with the α_5 integrin. Fibronectin matrix assembled by this integrin is primarily responsible for the suppression of malignant properties of cells expressing elevated levels of this integrin (Giancotti and Rouslahti, 1990; Rouslahti, 1996).

Since the intracellular domain of the β integrin sub-unit signals FAK phosphorylation, it is thus likely that integrins play an important role in regulating cell proliferation. The β_1 cytoplasmic domain has been shown to cause a stimulation in cell proliferation (Pasqualini and Hemler, 1994). A spliced β_1 subunit (β_1C) has been shown recently to be growth inhibitory, preventing cyclin A expression and DNA synthesis (Meredith *et al.*, 1995).

Role of integrins in apoptosis

Programmed cell death or apoptosis is a normal part of development, homeostasis, and the pathogenesis

of some diseases (Steller, 1995). There is evidence that integrin occupancy can trigger signals that inhibit apoptosis. This inhibition of apoptosis by the ECM is dependent upon the expression and function of particular integrin heterodimers, and these requirements appear to be cell type specific. Integrin ligation has been shown to regulate the expression of bcl-2, a key regulatory component in the suppression of apoptosis (Zhang *et al.*, 1995). Aoshiba *et al.* (1997) found that in cultured human bronchial epithelial cells, prevention of apoptosis by cell-cell adhesion is associated with bcl-2 expression and is mediated by the α_v integrin sub-unit. Loss of β_1 integrin contact with the ECM has been found to trigger the inhibition of G₁ cyclin-dependant kinase activity which results in dephosphorylation of the retinoblastoma tumour suppressor protein (Rb). This activation of Rb and its interaction of E2F can induce apoptosis in these cells. This apoptotic pathway is modulated by bcl-2 which can inhibit apoptosis by regulating Rb phosphorylation and hence its activity (Day *et al.*, 1997).

ECM degradation during normal development results in apoptosis of matrix-dependent cells (Re *et al.*, 1994). Primary cells of epithelial and endothelial origin rapidly undergo apoptosis if denied proper substrate adhesion, in a process that has recently been termed anoikis (Frish and Francis, 1994; Rouslahti and Reed, 1994). Inhibition of $\alpha_v\beta_3$ function during angiogenesis with an anti- $\alpha_v\beta_3$ antibody both inhibited proliferation and induced apoptosis whereas treatment with anti- β_1 antibodies did not (Brooks *et al.*, 1994). Howlett *et al.* (1995) reported that interruption of normal mammary morphogenesis by anti-integrin antibodies was associated with an inhibition of cell growth and induction of apoptosis.

Oncogenic transformation of rat primary fibroblasts with *myc/ras* or *E1A/ras* can lead to rapid cell death by apoptosis when ECM adhesion is prevented, i.e. by growing the cells in suspension. A soluble RGD-specific peptide can also trigger apoptosis in these cells suggesting integrin signalling events are involved in the process possibly via the $\alpha_5\beta_1$ or $\alpha_v\beta_1$ receptors (McGill *et al.*, 1997). On the contrary, activated *ras* oncogene has been found to protect the MDCK cell line from apoptosis on removal from the ECM (Khawaja *et al.*, 1997).

Apoptosis is suppressed in normal human melanocytes grown on fibronectin. However, when they are grown in suspension or on poly-L-lysine coated surfaces, apoptosis is induced (Scott *et al.*, 1997). This

effect appears to be mediated by the $\alpha_5\beta_1$ integrin as the use of blocking antibodies to this integrin enhanced apoptosis in these cells. Cytoskeletal organisation is required for mediating apoptotic signalling in melanocytes. Cytochalasin D, an inhibitor of cell spreading and cytoskeletal organisation, was found to severely disturb the protective effects of fibronectin on melanocyte apoptosis. This suggests that an intact cytoskeleton is required for transduction of survival signals (Scott *et al.*, 1997).

Engagement of specific β_1 integrins prevented apoptosis in mammary epithelial cells by inhibiting the expression of interleukin 1 β -converting enzyme (ICE), an inducer of apoptosis in mammalian cells (Boudreau *et al.*, 1995). Apoptosis was induced in neuroblastoma cells by using anti-sense β_1 integrin oligonucleotides and perturbing antibodies to β_1 integrin to inhibit cell-ECM interactions (Rozzo *et al.*, 1997).

Expression of the β_4 cytoplasmic domain in cells has been shown to activate p21 and induce growth arrest (Clarke *et al.*, 1995). The integrin sub-unit β_4 may be involved in apoptotic signalling in vascular endothelial cells (VEC). VEC cells when deprived of FGF and serum undergo rapid apoptosis which can be reversed by addition of anti- β_4 antibodies to the cells. It appears that the hemi-desmosome and keratin filament system might be important in the regulation of apoptotic signalling (Miao *et al.*, 1997).

There appears to be evidence that engulfment of apoptotic bodies by monocyte-derived dendritic cells is mediated by the $\alpha_v\beta_3$ integrin (Rubartelli *et al.*, 1997).

Role of integrins in tissue morphogenesis and differentiation

Epithelial branching morphogenesis is a process by which a continuous epithelium, embedded in mesenchyme, forms tubules and branches into surrounding mesenchyme. This is responsible for the architecture of many organs, including the lung. Integrin receptors may mediate the interaction between the ECM molecules and the epithelium to allow normal branching to occur (Gumbiner, 1996). There appear to be major changes in the expression of ECM molecules, such as laminins and integrins which may be important for normal lung development (Virtanen *et al.*, 1996).

The α_1 integrin has been found to be expressed on developing smooth muscle and endothelium. The α_3 and α_6 integrins are expressed pericellularly in young

epithelia, while as maturation progresses, these subunits become basally restricted. These integrins thus have roles in the maturation and polarisation of the epithelium (Wu and Santoro, 1996). The α_2 integrin is expressed at lung branch tips and may have a role in the formation of the basement membrane at growing tips or the extension of epithelia into surrounding mesenchyme (Virtanen *et al.*, 1996; Wu and Santoro, 1996;). *In vitro*, $\alpha_2\beta_1$ integrin has been found to play an important role in epithelial branching morphogenesis in mammary epithelial cells and kidney epithelial cells (Howlett *et al.*, 1995; Keely *et al.*, 1995; Zutter *et al.*, 1995).

Integrins must be functional for development to proceed normally. As the cells of an embryo proliferate and differentiate to form tissues of the body, they add and subtract integrins from their surface. Cells of the embryo also require integrins to help them travel to their final destination. 'Knocked-out' integrins often result in deformation or death during development (Fässler *et al.*, 1996; Horwitz, 1997).

Adhesion molecules generally vary with the epidermal stratification and differentiation states. Integrins appear to be extremely important in the regulation of events leading to terminal differentiation of keratinocytes (Jensen and Wheelock, 1996). Basal keratinocytes of the skin contain three populations based on proliferation capacity; stem cells, transit-amplifying cells and committed cells. Stem cells can be identified and sorted *in vitro* based on higher expression of β_1 integrins (Jones and Watt, 1993; Jones *et al.*, 1995). In committed cells, integrin function appears to be inhibited and they are lost from the cell surface. This allows keratinocytes to be free to move upwards through the epidermis from the basement membrane. There appears to be *in vitro* evidence that inhibition of integrin synthesis occurs only after the basal keratinocyte has begun to differentiate further and detach from the ECM. Expression of integrin receptors, especially α_2 , α_3 and α_5 , is down-regulated by forced suspension culture of keratinocytes (Adams and Watt, 1989) and may mimic detachment from the ECM *in vivo*. Symington and Carter (1995) have provided evidence that $\alpha_3\beta_1$ mediated adhesion to epiligrin (epidermal BM component) may be capable of regulating keratinocyte differentiation. They found that using anti- $\alpha_3\beta_1$ antibodies can inhibit keratinocyte adhesion to epiligrin and induce involucrin synthesis, a marker of terminal differentiation in keratinocytes.

The role of integrins in expression of markers of differentiation has been most extensively studied in mammary epithelial cells. When mammary cells are grown on plastic dishes, they cannot be induced to produce milk proteins. However in 3D culture, β_1 interaction with laminin results in activation of β -casein gene expression with resultant milk production (Streuli *et al.*, 1991). An ECM response element has been identified in the β -casein promoter (Schmidhauser *et al.*, 1992).

Regulation of Integrin Expression

The up- and down- regulation of certain genes in various cancers appears to be manifested at the transcriptional level. Given the involvement of integrin-mediated signals in regulating susceptibility to apoptosis, expression of integrins may be relevant to the response of cancer cells to chemotherapy. However, mechanisms controlling integrin gene expression are quite poorly understood and are only beginning to be elucidated (Kim and Yamada, 1997).

Mediators that regulate integrin expression

A number of growth factors and cytokines have been found to regulate the expression of integrins (see Tables II and III). Many tumours are heterogeneous populations with ECM heterogeneity within these tumours, which results in differences in integrin expression. Growth factors and cytokines also vary with tumour type and may directly or indirectly affect integrin expression. If tumour cells lose their ability to bind ECM proteins, growth factors such as TGF- α and FGF may exert growth stimulatory activities, due to loss of the normal regulatory control, mediated by ECM proteins, therefore inappropriate expression of growth factors seen in malignant tissue whose cell-matrix adhesion receptors are altered, may have deleterious effects on the maintenance of normal tissue architecture and growth (Pignatelli and Stamp, 1995). For example, TGF- β has also been found to upregulate certain integrins and downregulate others (see Tables II and III) through both transcriptional and post-transcriptional mechanisms (Loeser, 1997).

Adhesion of cancer cells to the vascular epithelium is an important step in the hematogenous metastasis of cancer. Heparin-binding EGF has been found to increase the expression of α_2 and α_3 integrins in human breast cancer cells (Narita *et al.*, 1996) and

Table 2. Upregulation of integrin expression by cytokines

Cytokine	Integrin	Cell type	Reference
FGF	α_1	rat PC12 cells	Zhang <i>et al.</i> (1993)
NGF	α_1	rat PC12 cells	Zhang <i>et al.</i> (1993)
EGF-R	α_2	cutaneous squamous carcinoma	Fujii (1996)
IFN- γ	$\alpha_1\beta_1$	monocytes	Rubio <i>et al.</i> (1995)
TGF- α	α_2, α_3	monocytes	Lin <i>et al.</i> (1994)
TGF- β	α_6, β_1	alveolar epithelial cells	Kumar <i>et al.</i> (1995)
	β_1	osteocarcinoma	Cervella <i>et al.</i> (1993)
TNF- α	$\alpha_1, \alpha_2, \alpha_5$	melanoma	Mortarini <i>et al.</i> (1991)
	α_2	bladder cancer	Nista <i>et al.</i> (1996)
IGF-1	$\alpha_1, \alpha_5, \beta_1$	chondrocytes	Loeser (1997)
IL-1 β	$\alpha_1, \alpha_2, \alpha_5$	melanoma	Mortarini <i>et al.</i> (1991)
TGF- β_1	β_1, α_3	melanoma	Mortarini <i>et al.</i> (1991)
	$\alpha_1, \alpha_2, \alpha_3, \alpha_5, \beta_1$	WI-38 fibroblasts	Heino <i>et al.</i> (1989)
	$\alpha_2\beta_1$	colon	Hafez <i>et al.</i> (1992)
	$\alpha_1\beta_1$	<i>ras</i> -transformed hepatocytes	Serra <i>et al.</i> (1994)
	α_5	chondrocytes	Loeser (1997)
	LFA-1	monocyte leukemia	Ignatz <i>et al.</i> (1989)

Table 3. Downregulation of integrin expression by cytokines

Cytokine	Integrin	Cell type	Reference
IFN- γ	$\alpha_2, \alpha_6, \beta_1$	lung cancer	Van Valen <i>et al.</i> (1994)
TNF- α	$\alpha_2, \alpha_6, \beta_1$	lung cancer	Van Valen <i>et al.</i> (1994)
	α_6	endothelial cells	Delfilippi <i>et al.</i> (1994)
IL-1 β	α_4	melanoma	Mortarini <i>et al.</i> (1991)
TGF- β_1	α_1	chondrocytes	Loeser (1997)
TGF- α	α_v, α_6	monocytes	Lin <i>et al.</i> (1994)
IFN- α	α_4	melanoma	Mortarini <i>et al.</i> (1991)

oesophageal cancer cells (Sato *et al.*, 1996) already expressing EGF-R. The interpretation from the findings of these groups is that cancer cells receive signals from the cytokines such as HB-EGF, produced by endothelial cells following initial adhesion of cancer cells *via* selectins. This causes a secondary increase in the expression of cell adhesion molecules and leads to augmentation in the adhesive activities of cancer cells at the vessel walls.

ECM and growth factors are important in morphogenesis and wound repair. In an *in vitro* model designed to mimic wound healing, Xu and Clark (1996) cultured normal human dermal skin fibroblasts in relaxed collagen gels and fibronectin-rich cultures or stressed fibrin gels, and stressed collagen gels. Integrin subunit mRNA levels were measured before and

after stimulation with PDGF-BB, a potent mitogen and chemoattractant for fibroblasts. The fibronectin-rich cultures and fibrin gel induced α_3 and α_5 mRNA expression. The stressed and relaxed collagen gels attenuated these responses while promoting maximal integrin α_2 expression. ECM alterations during wound healing or any new tissue formation causes cells to respond differently to repeated growth factor stimuli. During cutaneous wound repair *in vivo*, the provisional matrix of fibronectin, fibrinogen, fibrin and vitronectin forms in the wound area (Gailit and Clark, 1994). In the fibrin network, platelets release PDGF that stimulates proliferation and chemoattraction of fibroblasts (Deuel *et al.*, 1991). Activated fibroblasts must move from surrounding collagenous connective tissue into a fibrin/fibronectin-filled wound and subse-

quently synthesise new collagenous matrix. Thus fibroblast response to these ECM molecules and growth factors is essential for the healing process (Xu and Clark, 1996).

Several pharmacologic agents have been found to regulate integrin expression in various cell types. Bromodeoxyuridine (BrdU) has the ability to upregulate expression of α_3 , α_5 , and α_6 integrin levels in human melanoma cells (Thomas *et al.*, 1993) and α_v -associated integrins in neuroblastoma (Yoshihara *et al.*, 1997). Retinoic acid has been shown to alter β_1 expression in F9 teratocarcinoma cells (Ross *et al.*, 1994). The mouse embryonic stem cell line, P19, normally lacks the α_7 integrin but retinoic acid treatment of the cells induces differentiation to the smooth muscle cell lineage with a resultant high expression of the α_7 integrin (Yao *et al.*, 1997). Other agents such as phorbol esters (Ferreira *et al.*, 1991) and dexamethasone (Zhang *et al.*, 1993) can also regulate integrin levels in different cell types.

Control of integrin expression

Many of the α and β integrin subunits have been sequenced and cDNA clones are available for most of these. Ignatius *et al.* (1990) reported on the rat α_1 clone which is homologous to the human form of the α_1 subunit. The human α_1 structure has since been elucidated (Briesewitz *et al.*, 1993). Takada and Hemler (1989) cloned cDNA for the α_2 integrin gene. The rat α_1 and human α_2 subunit have been found to share greater than 50% sequence homology that may underline some of their similar ligand properties. The promoter regions of a number of integrin α subunits have been described and characterised (Birkenmeier *et al.*, 1991; Rosen *et al.*, 1991; Shelly and Arnaout, 1991; Uzan *et al.*, 1991; Corbi and Lopez-Rodriguez, 1997; Kim and Yamada, 1997; Lin *et al.*, 1997). A common feature demonstrated by these promoters is the lack of TATA and CCAAT boxes and high G+C content, with the exception of the α_4 (Rosen *et al.*, 1991), α_5 (Birkenmeier *et al.*, 1991) and α_6 (Lin *et al.*, 1997) integrin promoters. All the characterised integrin gene promoters drive the expression of reporter genes in a tissue-specific manner, which corresponds to the tissue-specific expression pattern of the respective genes (Ziober *et al.*, 1996).

The promoter region of α_2 integrin seems to be the most characterised and was found to lack TATA and CCAAT boxes, but contains an abbreviated initiator sequence and Sp1 binding sites. Consensus binding

sequences for AP-1 and AP-2 complexes, a Pu-1 box, a GATA, and 2 palindromic motifs with potential to bind the oestrogen receptor are also present (Zutter *et al.*, 1994). Two Sp1 binding sites in the core promoter of the α_2 gene located between bp -30 and -92 are required for full promoter activity and for DNA-protein complex formation (Zutter *et al.*, 1997). The α_6 integrin gene promoter has only recently been characterised (Lin *et al.*, 1997) and has been found to contain Sp1 and AP-2 binding sequences. It also has a possible epithelial cell binding sequence, Ker 1, which was first identified as a keratinocyte-specific transcription factor in the human keratin 14 gene promoter (Leask *et al.*, 1990).

The regulatory region of the human β_1 gene consists of two promoters tandemly located which appear to be independently regulated. The two promoters drive the expression of the unique β_1 -gene resulting in the synthesis of at least two mRNAs, that diverge only in the complete 5'-untranslated region and share the same coding sequence, as is indicated by the isolation of two cDNAs with this structure. The longer mRNA transcript is ubiquitously present in human tissues and is 20-fold more abundant than the shorter transcript, which is expressed only in a subset of tissues (Cervella *et al.*, 1993).

An octamer sequence (Oct 185) has recently been identified as a regulatory element in the α_x (CD11c) promoter whose disruption leads to an increase in the basal activity of the α_x promoter. This sequence has been found to be differentially recognised by the POU/homeodomain transcription factors Oct-1 and Oct-2. Oct-2 and α_x expression are concomitantly induced during monocyte differentiation (Lopez-Rodriguez *et al.*, 1997). PU.1 is a member of the ETS family of transcription factors which has tissue-specific activity in the lymphoid and myeloid lineages (Klemz *et al.*, 1990) and has been found to have a negative repressor activity on the α_x gene promoter at the major transcriptional start site PUI-5 (Lopez-Rodriguez and Corbi, 1997). However, it has been found to show positive regulation of the α_m (CD11b) promoter activity in myeloid cells (Pahl *et al.*, 1993).

N-myc has been found to modulate the expression of some integrin gene promoters. Transfection of N-myc into human neuroblastoma cells (Judware and Culp, 1997a) and osteosarcoma cells (Judware and Culp, 1997b) results in reduced levels of α_2 , α_3 and β_1 protein levels in neuroblastoma cells and α_3 and β_1 protein levels in the osteosarcoma cells. The mRNA

for the α_2 and α_3 integrin was found to be reduced suggesting transcriptional control of these integrin gene promoters by N-myc. However, β_1 integrin mRNA levels were found only to be slightly reduced suggesting some post-transcriptional control by N-myc. The cells in both cases were found to adhere very poorly and had a more rounded morphology compared to control untransfected cells. The most advanced stages of neuroblastoma show an overexpression of the N-myc oncogene (Bernstein *et al.*, 1992). The β_1 integrin promoter has an N-myc consensus binding site (Cervella *et al.*, 1993) as well as a non-consensus binding site (Blackwell *et al.*, 1993). Two non-consensus sites have been located on the α_2 gene promoter (Blackwell *et al.*, 1993). The α_3 integrin gene promoter has not yet been characterised. N-myc appears to be exerting a negative regulatory effect on transcription from some integrin gene promoters.

Several integrin promoters contain retinoic acid response elements, including α_m (Hickstein *et al.*, 1992) and β_2 (Agura *et al.*, 1992).

Integrins and cancer

Integrins are required for tissue morphogenesis and disruption of expression or function may lead to loss of differentiation of malignant cells and their ability to invade normal tissue (Albelda, 1993; Pignatelli and Vessey, 1994). It appears that there is no single integrin expression profile that correlates with malignancy. Rather, for different types of cancer, different stages in integrin expression seem to be associated with transformation and tumour progression (Pignatelli and Stamp, 1995). However, there appears to be a trend for the down-regulation of β_1 integrins in epithelial carcinomas (Ziobar *et al.*, 1996).

The $\alpha_2\beta_1$ integrin is consistently lost or decreased in moderately and poorly differentiated epithelial tumours as described in Table IV. The potential tumour suppressor function of this integrin has been shown in breast cancer where antisense mRNA reduction of $\alpha_2\beta_1$ integrin induced a transformed phenotype (Keely *et al.*, 1995) while transfection of this integrin into a poorly-differentiated mammary carcinoma was found to restore the ability of the cells to differentiate into gland-like structures in 3D matrices (reconstituted Matrigel) and markedly reduced their *in vivo* tumorigenicity (Zutter *et al.*, 1995).

In some cancers, high levels of the $\alpha_2\beta_1$ integrin has been associated with metastasis. This integrin may

Table 4. Loss of α_2 integrin in poorly differentiated carcinomas

Tumour type	Reference
Colorectal	Koukoulis <i>et al.</i> (1993)
	Nigam <i>et al.</i> (1993)
	Pignatelli <i>et al.</i> (1990)
	Koretz <i>et al.</i> (1991)
Oral carcinoma	Kosmehl <i>et al.</i> (1995)
NSCLC	Damjanovich <i>et al.</i> (1992)
SCLC	Pellegrini <i>et al.</i> (1994)
Breast cancer	Zutter <i>et al.</i> (1995)
	Koukoulis <i>et al.</i> (1991)
	Arihiro <i>et al.</i> (1993)
	Pignatelli <i>et al.</i> (1991)

play a dual role in poorly differentiated epithelial tumours by inhibiting early stages but facilitating late events in the metastatic process (Danen *et al.*, 1995a). Highly metastatic melanoma cells have been found to over-express this integrin (Klein *et al.*, 1991; Albelda *et al.*, 1990). Transfection of this integrin into a rhabdomyosarcoma cell line resulted in a more metastatic cell type that showed increased adhesion to collagen and laminin (Chan *et al.*, 1991). Chen *et al.* (1994a) looked at the integrin expression in various clones from a human NSCLC primary tumour. Parental cells expressed low amounts of α_1 , α_2 and β_1 integrins. However, one of the clones was found to express high amounts of the α_1 and α_2 integrins. Different clonotypes from the same tumour may have different invasive and metastatic potentials. In primary anal carcinomas, no α_2 integrin expression was detected but cells from metastatic tissue showed α_2 positivity (Anastassiou *et al.*, 1995).

Loss of α_1 integrin has been observed in lung adenocarcinomas, compared to high expression in normal lung pneumocytes (Roussel *et al.*, 1994). Down-regulation of α_3 integrin has been observed in NSCLC (Smythe *et al.*, 1995; Chen *et al.*, 1994b), breast carcinoma (Pignatelli *et al.*, 1991) and colorectal carcinoma (Pignatelli *et al.*, 1990). In CHO cells, a clone expressing low levels of $\alpha_3\beta_1$ grew most rapidly in nude mice while cells with elevated levels of the integrin grew much more slowly (Schreiner *et al.*, 1991). Transfection of $\alpha_3\beta_1$ integrin into rhabdomyosarcoma also repressed the transformed phenotype (Weitzman

et al., 1996). The $\alpha_5\beta_1$ integrin can have both positive and negative effects on malignant progression. Elevated levels of $\alpha_5\beta_1$ integrin in CHO resulted in a suppression of the transformed phenotype (Giancotti and Rouslahti, 1990). However, transfection of the α_5 integrin into a human colon carcinoma cell line rendered the cells more tumourigenic (Gong *et al.*, 1997).

Up-regulation, down-regulation and loss of α_6 integrin has been implicated in tumour progression and metastasis in many cell types. The upregulation of this integrin appears in tumours where normal tissue express low or undetectable levels (see Table V). The down-regulation of α_6 has been implicated in epithelial tumours that normally express high levels of this integrin (see Table V). In lung cancer, the results are somewhat variable but high $\alpha_6\beta_4$ expression has been shown in highly metastatic Lewis lung carcinoma (Perrotin *et al.*, 1990) and SCLC (Feldman *et al.*, 1991) while low levels of expression have been reported in NSCLC (Smythe *et al.*, 1995). The difference in expression may be due to the fact that mesenchymal cells (*e.g.* fibroblasts) have no need to interact with BM and thus have no need to express α_6 integrin but when the cells become transformed they must interact with BM to invade tissue, hence increased expression. In epithelial cells which are associated with BM, for carcinomas to detach from the BM, a reduction in α_6 expression may be necessary but on development of the secondary metastasis, expression of α_6 may be required for attachment to the BM and to initiate invasion. A ribozyme that selectively degrades α_6 integrin mRNA was transfected into a human fibrosarcoma cell line and resulted in a change to a less adherent and less invasive cell type (Yamamoto *et al.*, 1996).

Integrins have been found to have a role in angiogenesis of tumours. To form new blood vessels, proliferating endothelial cells must form adhesive attachments to one another and to the matrix around them. As a result there is often increased expression of the $\alpha_v\beta_3$ integrin associated with invasive cancers. This has been observed in metastatic melanomas (Albelda *et al.*, 1990; Danen *et al.*, 1995a; Natali *et al.*, 1997), colorectal carcinoma (Nigam *et al.*, 1993), and breast carcinoma (Pignatelli *et al.*, 1992).

There appears to be an association of integrin expression with expression of metalloproteinases (MMPs) (Heino, 1996). MMPs appear to play an important role in cancer cell invasion and metastasis (Liotta, 1986; Ura *et al.*, 1989). Collagenase MMP-2

Table 5. Changes in α_6 integrin expression in cancer

Tumour type	Reference
Upregulation of α_6	
Fibrosarcoma	Lin <i>et al.</i> (1993) Yamamoto <i>et al.</i> (1996)
Melanoma	Danen <i>et al.</i> (1995b)
Liver cancer	Volpes <i>et al.</i> (1993)
Skin cancer	Tennenbaum <i>et al.</i> (1993)
Downregulation of α_6	
Breast	D'Ardenne <i>et al.</i> (1991)
Colon	Stallmach <i>et al.</i> (1992)
Prostate	Knox <i>et al.</i> (1994)
Kidney	Korhonen <i>et al.</i> (1992)
Pancreas	Weinel <i>et al.</i> (1995)

has been shown to bind directly to $\alpha_v\beta_3$ and is thus localised in proteolytically active form on the surface of invasive tumour or endothelial cells (Brooks *et al.*, 1994; Seftor *et al.*, 1993). Clarke *et al.* (1997) have shown that production of MMPs is associated with increased α_v expression. There appears to be an association between $\alpha_2\beta_1$ integrin expression and positive regulation of MMP-1 (Riikonen *et al.*, 1995). Using antibodies to α_3 integrin resulted in increased levels of MMP-2 and greater invasive capabilities in Matrigel assays of glioblastoma cells (Chintala *et al.*, 1996).

The onset of multiple drug-resistant phenotypes in tumour cells has been found to be associated with altered expression of adhesion and cytoskeletal components (Biedler *et al.*, 1991; Rivoltini *et al.*, 1991). Exposure of a renal carcinoma cell line to vinblastine was found to be associated with an acquisition of $\alpha_1\beta_1$ and $\alpha_2\beta_1$ integrins and a reduction in $\alpha_6\beta_1$ integrin expression with an associated increased level of adhesion of the drug-resistant cells to type I and type IV collagens and to fibronectin (Duensing *et al.*, 1996). Nista *et al.* (1997) have shown induction of $\alpha_4\beta_1$ and $\alpha_5\beta_1$ integrin protein and mRNA in Adriamycin-resistant MCF-7, breast carcinoma cells (ADR^R MCF-7) compared to the sensitive parental cell line. The presence of the $\alpha_5\beta_1$ receptor on ADR^R MCF-7 cells confers on them a dependency on fibronectin in order to allow cell survival and prevent them from entering an apoptotic pathway (Nista *et al.*, 1997).

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