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# Reciprocal Paracrine Interactions Between Tumour Cells and Endothelial Cells: the 'Angiogenesis Progression' Hypothesis

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## ON GENES, TISSUE GEOMETRY AND ANGIOGENESIS PROGRESSION

IT IS currently believed that solid tumours emerge, in part, as a consequence of the loss of spatial information encoded by the developmental programme of the affected tissue. In normal intestinal mucosa, for example, both cell number and position are tightly controlled in order to maintain the physiological sheath-like (quasi two-dimensional) cell topology [1]. In this case, homeostasis is maintained through multiple levels of regulation of such cellular processes as proliferation, differentiation and programmed cell death [1, 2], as well as through intercellular interactions and proper response to varying microenvironmental conditions. In transformed cells, these control mechanisms are gradually compromised, bypassed or 'highjacked' in order to allow an unrestricted (three-dimensional) tumour growth.

### *Molecular determinants of the three-dimensional tumour growth in colorectal carcinoma*

The realisation of various functional requirements that need to be fulfilled in order for unrestricted tumour growth to proceed, naturally leads to the inquiry about their molecular basis. Colorectal cancer was the first human malignancy where multiple quasi-sequential genetic alterations were characterised in depth, and linked to clinical stages of disease progression [3]. Initially, little was known about the functional role and contribution of each particular aberrant gene involved in the process. This situation has recently begun to change, providing the first suggestions that not only abnormalities in cellular mitogenesis but also in cell survival and angiogenesis are involved in the development of three-dimensional tumour growth 'geometry'.

One of the earliest genetic changes known to occur in colorectal cancer is loss-of-function mutation of the gene known as *APC* (*adenomatosus polyposis coli*). While its exact function still remains a mystery, recent findings suggest an association of the *APC* gene product with intracellular catenins raising the possibility that *APC* may relay the growth regulatory signals from cell surface adhesion molecules to their intracellular targets [4]. Interestingly, *APC* may also have an impact on the regulation of local blood vessel formation and homeostasis. This can be inferred from the fact that in *Min* mice, which harbour a mutant *APC* homologue, a major cause of lethality is the haemorrhage from large benign intestinal polyps that these mice develop [4]. Recent advances in our understanding of biochemical signalling pathways have imparted a new meaning to the fact that activating mutations of the *K-RAS* proto-oncogene are amongst the most common genetic changes detected in colon cancer [4]. It is becoming clear that the central role of the *RAS* pathway, in cellular signalling and gene expression control, is responsible for a major pleiotropic change in cellular phenotype and for selective growth advantage of cells harbouring such a mutant gene [5]. Thus, constitutively activated (GTP bound) ras protein may, in a permissive context of other molecular changes, switch the cell into a permanent 'growth mode' due to its combined effect on cell proliferation [6, 7], survival [8] and angiogenic competence [9, 10]. This 'single gene–triple hit' scenario represents an interesting paradigm from the cancer progression viewpoint and, therefore, shall be discussed in more detail in the next section.

Another example of a genetic lesion possibly involved in three-dimensional growth, as it relates to a defective control of tissue geometry, is the loss of *DCC* (deleted in colorectal carcinoma) tumour suppressor gene [4]. This is because it is now believed that *DCC*, like its analogue *UNC40* in *C. elegans*, may be a receptor for the morphogenic peptide called netrin and thereby may participate in spatial control of cellular growth and differentiation (J. Culotti, S. Lumenfeld Institute, Mt Sinai Hospital, Toronto, Ontario, Canada). A similar notion has been recently proposed in relation to the elements of the cell cycle machinery, and in particular to certain cyclin dependent kinase inhibitors (CKIs) [2, 11]. Thus, the CKI known as $p21^{WAF1}$, was found to be expressed by colonic epithelium in a cell position-dependent manner [2]. In contrast, no such topological restriction of $p21^{WAF1}$ expression is observed in colorectal adenomas [2]. Based on this and other recent findings, it is now suspected that $p21^{WAF1}$, $p27^{Kip1}$ and perhaps other CKIs may play a role in some aspects of tumour cell survival [11–15].

An obvious consequence of three-dimensional tumour growth is a continuous increase in distances between tumour cells and the existing vasculature. This is particularly so at the later stages of tumour growth and may result

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in poor perfusion of such large tumour masses, as well as metabolic stress, growth factor deprivation and hypoxia. Mutations of the *TP53* tumour suppressor gene are found more frequently at the later stages of colon cancer progression and may contribute to three-dimensionality of solid tumour expansion in many different ways. Loss of function in the case of *TP53* may, for example, impact on the G1 cell cycle checkpoint, deregulate cellular response to hypoxia [16, 17] and promote angiogenesis via its regulatory effect on expression of vascular endothelial growth factor/vascular permeability factor (VEGF/VPF), the angiogenesis inhibitor—thrombospondin (TSP-1), and possibly other relevant genes [18–21]. Furthermore, the notion that *TP53* mutations could facilitate enhanced survival of tumour cells in hypoxic conditions raises the possibility that such p53 mutant cells may remain intact as a source of angiogenic factors, rather than being eliminated by hypoxia-induced apoptosis [16, 17]. In fact, such viable tumour cells would probably up-regulate VEGF production due to hypoxia-dependent increases in the level of VEGF gene transcription, and mRNA stability [22–27]. The latter effect may contribute to the overall intensity of angiogenesis at the later stages of colon cancer progression. Moreover, genetic instability, which is associated with mutations of not only *TP53* but also other recently cloned genes, particularly those involved in non-polyposis colorectal carcinoma (HNPCC) such as *hMSH2* and *hMLH1* [4], may act as a higher order defect to influence molecular mechanisms of three-dimensional growth and angiogenesis. Identification of the angiogenesis effector genes in the case of HNPCC is a currently intriguing question of this area of research. Nevertheless, it is becoming clear that the 'angiogenic switch' during progression of colorectal carcinoma, and most likely other solid tumours, may not be confined to a single event (like tumour progression itself). Rather, tumour angiogenesis may be the consequence of a multistep process parallel to (and probably caused by) a sequence of accompanying genetic alterations in tumour cells [4, 28, 29] (Figure 1).

*Impact of genetic defects associated with tumour progression on angiogenic properties of cancer cells*

Angiogenesis is clearly a central requirement for an unrestricted growth of multiplicity of solid tumours [30]. Not just *TP53* mutations but also losses of other tumour suppressor genes, e.g. *VHL* and *Lod2* (locus in RIP-Tag mice) or activation of oncogenes including *RAS*, *RAF*, *FOS*, *SRC*, *NEU*, *BPV*, *SV40Tag* may all have an immediate impact on production by tumour cells of various angiogenesis regulators [9, 10, 18, 20, 21, 31–38]. These may include, for example, VEGF, bFGF (basic fibroblast growth factor), TSP-1 or glioma derived angiogenesis inhibitory factor [18, 21, 34, 37]. Through pleiotropic effects on gene expression [18, 37, 39], signalling and cellular stress responses [38, 40], mutant oncogenic proteins may precipitate cytokine networks and unleash enzymatic cascades [39] potentially capable of influencing various aspects of angiogenesis. Depending on the type, timing and combination of these genetic defects, the biological forces driving tumour angiogenesis may change both quantitatively and qualitatively. A stepwise accumulation of genetic alterations during tumour progression is likely to be paralleled by a stepwise change (increase) in angiogenic competence of tumour cells followed by a sequential modifications in gene expression pattern, phenotype and behaviour of tumour associated endothelial cells. This hypothetical, genetically driven, and continuously evolving pattern of tumour-endothelial cell interactions will be referred to in the remainder of this review as malignant '*angiogenesis progression*'

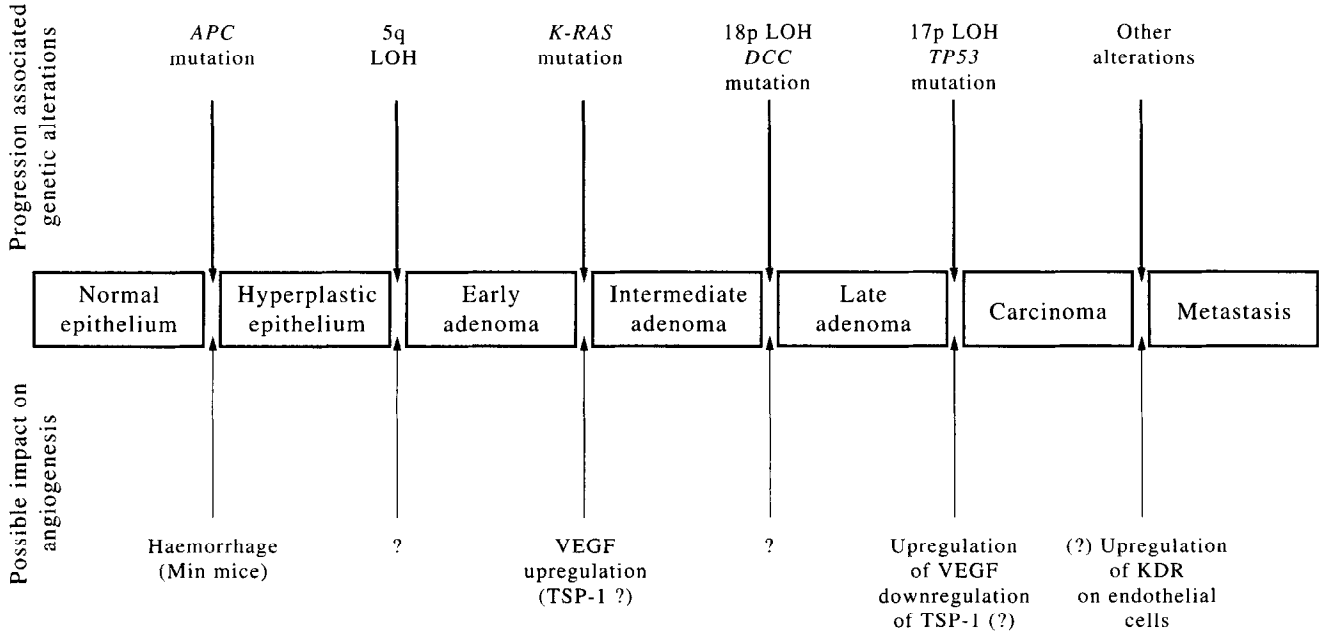


**Figure 1. Parallel between known genetic alterations during progression of colorectal carcinoma and postulated changes in tumour angiogenesis profile. Expression of some of the angiogenesis stimulators and inhibitors may be influenced directly or indirectly by sequential genetic events involving oncogenes and tumour suppressor genes, growth factors and microenvironment (see text; adapted from references [4, 18, 28, 29, 39]).**

## MUTANT *RAS* ONCOGENES, VEGF, ANGIOGENESIS AND METASTASIS

*Consequences of RAS mutations for the phenotype of transformed intestinal epithelial cells*

The exact biological 'algorithms' by which different types of tumour cells acquire the ability to recruit blood vessels effectively are, in all likelihood, complex and multifactorial. In general, they are thought to involve changes in the balance between various paracrine angiogenesis stimulators and inhibitors produced by parenchymal, stromal and/or inflammatory cells at the tumour site [41–46] (Table 1).

In order to frame the regulation of angiogenesis in the context of molecular alterations driving tumour progression, it would be instructive to examine more closely a simplified (but undoubtedly realistic) case where the expression of a single oncogene, such as mutant *H-* or *K-RAS* is closely correlated with the ability of transformed cells to grow progressively *in vivo*, and hence should clearly involve some aspects of angiogenesis.

Mutations of *RAS* proto-oncogenes are relatively common in cancer and are found in approximately 50% of colorectal cancer patients (predominantly of the *K-RAS* isoform) [4, 48, 49]. In this group of patients, *K-RAS* mutations tend to become detectable around the time of transition from early to intermediate premalignant adenoma stages of the disease. This transition usually coincides with a rapid three-dimensional expansion of the tumour mass [4]. In fact, mutant *K-RAS* is rather uncommon in tumours smaller than 10 mm in diameter [4]. Furthermore in 'flat' or superficial type colorectal adenomas, which do not form exophytic outgrowths, *K-RAS* mutations are 2–3 times less frequent than in the case of three-dimensionally growing polypoid tumours [48, 49]. These observations suggest that selection of tumour cells for *K-RAS* mutations may be required for, or at least facilitate, the three-dimensional tumour growth 'geometry'. This is in keeping with experimental data showing that, in colorectal cancer cell lines harbouring a single mutant *K-RAS* allele, somatic knockout of that allele leads to abrogation of anchorage independent growth in soft agar and to a complete [50] or partial [51] abrogation of the ability of these knockout cells to form tumours in nude mice. These *RAS* knockout cells also display a moderate decrease in proliferative activity in monolayer culture [50]. The reverse approach, i.e. induced overexpression of mutant *RAS* in non-tumorigenic cells, has been shown, by numerous studies, to produce malignant transformation of different cell types. In immortalised intestinal epithelial cells, IEC-18, such a modification endows the cells with an aggressive, tumorigenic phenotype *in vivo*, with a colony forming ability in soft agar, and an approximately 25% decrease in cell turnover time in monolayer culture [52].

It was originally assumed that the transforming properties of *RAS* oncogenes are mainly associated with their ability to stimulate cell mitogenesis directly. This notion has recently been substantiated by the finding that constitutive activation of *RAS* leads to perturbations in cell cycle control, notably through constitutive upregulation of cyclin D1 [53, 54]. However, in the context of colorectal cancer, accelerated cellular proliferation may not be the only relevant consequence of *K-RAS* mutation. This supposition is based on some earlier studies which point out that the average cell turnover time of tumour cells is not significantly shorter than that of dividing cells in corresponding normal mucosa [55]. In light of this observation, additional factors contributing to *in vivo* growth need to be considered. Thus, it is interesting to note that development of colorectal tumours has been recently associated with a stepwise decrease in rate of tumour cell apoptosis [56]. This important finding raises the distinct possibility that mutant *RAS* may play a causative role in aborting programmed cell death in transformed intestinal cells. Such a view is, in fact, supported by results of our experiments with the IEC-18 rat intestinal epithelial cell line. IEC-18 cells grow readily (and survive) in monolayer culture, but when deprived of adhesive substrate, and forced to assume a three-dimensional (spheroid) or suspension configuration, they rapidly undergo massive programmed cell death [8]. Transfection of IEC-18 cells with oncogenic *RAS* (or *SRC*) abrogates this apoptosis-like process, which we called 'adhesion regulated programmed cell death' (ARPCD). Both the mitogenic activity and cell viability under such restrictive three-dimensional culture conditions tend to correspond to the level of activated *H-RAS* protein that is expressed by the transfected cells [8]. Partial resistance to ARPCD could also be achieved in IEC-18 cells by enforced overexpression of human *BCL-2* proto-oncogene; however, in the latter case, the cells are unable to form tumours in nude mice (Mitsuhashi, Rak and Kerbel, Sunnybrook Health Science Center, Ontario, Canada). In contrast, *H-RAS* transfected cells form rapidly growing, highly vascular (haemorrhagic) tumours in nude mice, implying that, in addition to morphological features of transformation, a higher rate of proliferation and 'three-dimensional survival', they also acquired an angiogenic phenotype.

*Table 1. Examples of molecular control of tumour angiogenesis*

| Observation | Angiogenic effector molecule | References |
|---|---|---|
| Onset in production of angiogenic factor by human melanoma | bFGF | [106] |
| Onset in secretion of angiogenic factor by fibrosarcoma harbouring *BPV* transgene | bFGF | [34] |
| Loss of angiogenesis inhibitor in cells lacking *TP53* tumour suppressor gene | TSP-1, GD-AIF | [21, 120] |
| Upregulation of angiogenic growth factor upon loss of *VHL* tumour suppressor gene | VEGF | [121, 122] |
| Altered balance of angiogenesis stimulator and inhibitors in pancreatic islets of RIP-Tag mice | VEGF, bFGF, other? | [32, 33, 115] |
| Upregulation of angiogenic activity in cells transformed with *RAS*, *RAF* and *SRC* oncogenes | VEGF↑ (TSP-1↓) | [9, 10] |

Adapted from [47].

*Mutant RAS-mediated 'angiogenic switch'*

Oncogenic *RAS* may influence expression of a number of genes, some of which may be relevant for angiogenesis [39, 47]. Various growth factors, proteolytic enzymes and extracellular matrix (ECM) proteins have been shown to be regulated by *RAS* genes in a manner that is sometimes referred to as a 'masterswitch effect' [39, 47, 57]. It is not entirely clear what the relative contribution of each of these different genes is to the *RAS* dependent 'angiogenic switch'. In IEC-18 cells, for example, overexpression of the *H-RAS* oncogene led to significant upregulation of at least two potentially angiogenic peptide growth factors, namely TGF-α (transforming growth factor-α) [58] and VEGF [9]. In all probability, the latter factor is more directly involved in the angiogenic and tumorigenic properties of *RAS* transformed IEC-18 cells. This is because addition of the TGF-α neutralising antibody had no effect on the activity of the IEC-18/ras (clone RAS-7) conditioned medium when tested against human umbilical endothelial cells (HUVEC). More specifically, regardless of whether the antibody was present or not, addition of RAS-7 conditioned medium to growth factor-starved cultures of HUVECs stimulated thymidine incorporation by these cells and prevented them from undergoing apoptotic death (Rak and Kerbel, Sunnybrook Health Science Center, Ontario, Canada). In contrast, addition of anti-VEGF antibody significantly reduced the HUVEC stimulating activity of such tumour cell conditioned medium [9]. These observations are in agreement with the known angiogenic potency and endothelial cell specificity of VEGF, and support its role as the major pro-angiogenic growth factor that is upregulated transcriptionally by oncogenic *RAS*. They also argue against the proposition that the effect of mutant *RAS* on VEGF transcription is mediated exclusively by a TGF-α autocrine loop, at least in the IEC-18 system. Interestingly, mutant *RAS* oncogene also appears to be responsible, in some cases, for hypersensitivity of tumour cells to hypoxia-induced VEGF expression [149]. Another factor that might contribute to angiogenic and tumorigenic properties of various clones of *H-RAS* transfected IEC-18 cells, is the significant reduction in expression of the known angiogenesis inhibitor, TSP-1, in these cells (Rak, Kerbel, Sunnybrook Health Science Center, Ontario, Canada).

*The relationship between angiogenic competence, cell survival and metastatis of RAS-transformed epithelial cells*

Even though angiogenesis is undoubtedly a prerequisite of tumour metastasis, there seems to be no simple correlation between the levels of VEGF (or TSP-1) in various *RAS* transfectants of IEC-18 cells and their metastatic ability. In fact, only the cell line expressing the highest levels of *RAS* (clone RAS-7) gave rise to relatively small numbers of macroscopic metastases in the lungs of mice bearing subcutaneous tumour implants (Figure 2). By comparison, overt metastases or even single clonogenic cells were rarely detectable in lungs of mice bearing RAS-3 tumours, a clone expressing relatively lower levels of H-ras protein compared with RAS-7 cells. When mosaic tumours were generated by injecting the RAS-7 and RAS-3 cell mixtures, RAS-7 cells still displayed a metastatic and growth dominant phenotype, while the presence of RAS-3 derived metastases or single clonogenic tumour cells in lungs remained largely undetectable. In these experiments, RAS-7 and RAS-3 cells were

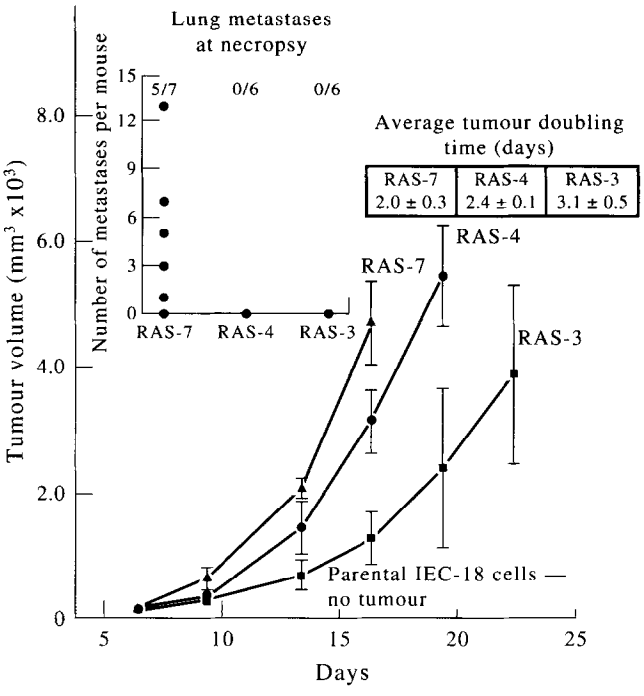


**Figure 2. Tumorigenic and metastatic properties of *H-RAS* transformed clones of the intestinal epithelial cell line, IEC-18. RAS-7 cells are the highest *H-RAS* expressors, while RAS-3 express relatively lower levels of exogenous Ras protein and RAS-4 is an intermediate variant. All the cell lines are highly tumorigenic, angiogenic and VEGF producing. Only RAS-7 clone gives rise to small numbers of macroscopic metastases in lungs of tumour bearing mice. In the case of RAS-4 individual tumour cells, but not macroscopic lung nodules, were present. Small numbers of clonogenic tumour cells were only occasionally detected in lungs of RAS-3 tumour bearing mice.**

injected at 1:4 ratio in order to compensate for their inherently different *in vivo* growth rates. Interestingly, differences were detected in the distribution of both RAS-3 and RAS-7 cell populations in different regions of the primary tumour. The cellular composition of 'proximal' (perivascular) and 'distal' (hypoxic) regions was evaluated in such mosaic primary tumours by injecting tumour bearing mice with a non-saturating concentration of the fluorescent, DNA binding dye known as Hoechst 33342. This dye is known to form perivascular gradients and has been previously used for analysis of drug penetration from the vascular compartment into tumour parenchyma [59]. In our experiments, individual mosaic tumours were removed 20 min after injection, dispersed by gentle enzymatic treatment and the recovered cells were sorted by FACS into two fractions representing either 'dim' i.e. hypoxic (distal) region or 'bright' cells from perivascular region (Figure 3). The clonogenic cells within each population were identified as RAS-7 or RAS-3 based on their colony forming ability in the selective media containing either G418 and thioguanine (against which RAS-7 cells are resistant) or G418 and HAT (in which RAS-3 but not RAS-7 cells were able to grow). Interestingly, viable clonogenic RAS-3 cells were usually less abundant throughout the tumour and mainly detectable within the perivascular region. In contrast, clonogenic RAS-7 cells were found in both perivascular and hypoxic areas (Figure 3). This may suggest that RAS-7 cells (i.e. *H-RAS* high expressors) have

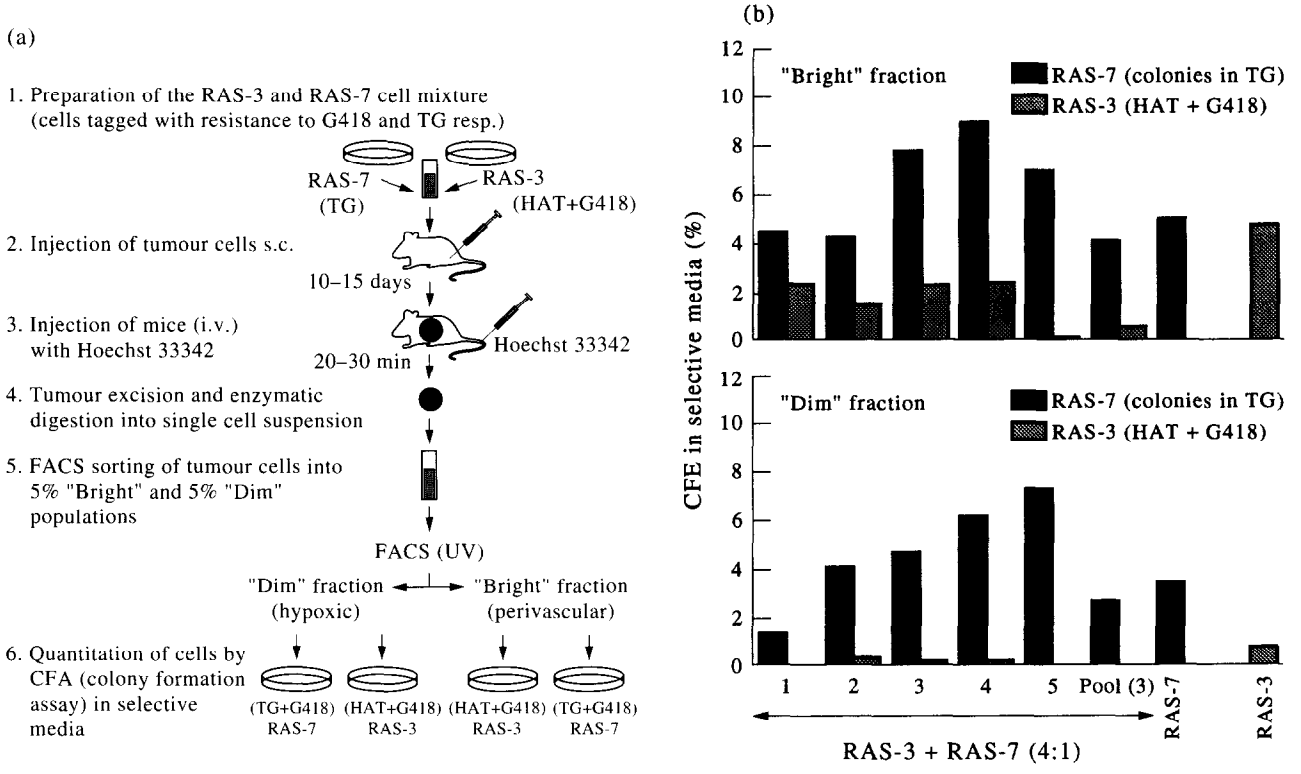


**Figure 3. Cellular composition of the perivascular and avascular compartments of mosaic tumours containing RAS-3 and RAS-7 populations. (a) Experimental design. Mixtures of RAS-3 and RAS-7 cells (4:1) were injected into the flank region of nude mice and grown to approximately 10 mm in diameter (10–15 days). At that time, a solution of the non-toxic, DNA binding dye known as Hoechst 33342 was injected intravenously to tumour bearing mice and allowed to diffuse into tissues in order to form a perivascular concentration gradient (for approximately 20 min) [59]. Immediately thereafter, the mice were sacrificed, tumours excised and dispersed into a single cell suspension using a cocktail containing collagenase IV, collagenase I, elastase and hyaluronidase. Cell suspensions obtained from several tumours were sorted by FACS individually, or as a pool into 5% of the 'dim' and 5% 'bright' populations, each containing certain proportions of RAS-3 and RAS-7 cells. The cells were immediately plated in selective media (G418/HAT for RAS-3 cells; G418/Thioguanine(TG) for RAS-7 cells) and colony forming efficiency (CFE) was calculated for each cell line as a percentage of cells that gave rise to clonal colonies within 10 days. (b) In the Hoechst 33342 positive, 'bright' i.e. perivascular fraction of mixed tumours, both RAS-3 and RAS-7 cell populations were represented albeit at different numbers. In the 'dim' fraction, which stained less intensely with the dye being at the greater distance from the microvessels, the RAS-3 cells were underrepresented or absent. This implies that the 'dominance' of metastatic RAS-7 cells is more pronounced in the hypoxic centres of tumour microdomains. In both fractions 'pure' RAS-3 and RAS-7 tumours gave the expected patterns of CFE in selective media.**

a greater survival ability under hypoxic conditions as they do in the previously described spheroid culture model [8]. Collectively, these observations imply that, in addition to angiogenic competence, enhanced resistance to stress may be required for full expression of the metastatic phenotype by tumour cells [8, 60].

*Regulation of angiogenesis by various components of the RAS signalling pathway*

From the above discussion, it appears that in some cases a single mutant oncogene, such as *H-RAS*, can simultaneously have an impact on cell proliferation, survival and angiogenesis. Moreover, mutational changes in ras protein structure may not be necessary for constitutive activation of the *RAS* signalling pathway. Prolonged upstream signals from *SRC*, EGFR or neu/ErbB2 kinases may lead to similar (albeit not identical) functional consequences [61, 62], including induction or upregulation of angiogenesis. Perhaps it is not a coincidence that in certain types of human tumours both overexpression of the *c-erbB2* proto-oncogene and high blood vessel counts are among the most significant prognostic indicators [63]. It is conceivable that activation of *ERBB2/NEU* or EGFR may be responsible for upregulation of VEGF (and other relevant angiogenic factors) by certain types of tumour cells. Furthermore, it is tempting to speculate that the impact on angiogenesis may be one of the plausible causes of why monoclonal neutralising antibodies against EGF and c-erbB2 receptors may sometimes be more potent as anti-cancer agents *in vivo* than they are in *in vitro* growth inhibition experiments [64, 65].

Both therapeutic and biological considerations lead to the inquiry about the downstream effectors of ras responsible for the 'angiogenic switch' and VEGF overexpression. It has been shown in this regard that, in some cell types, the raf/MEK/MAPK signal transduction pathway may play a major role, as fibroblasts can be transformed with activated forms of *RAF* or *MEK*-1 kinases and form tumours in mice [66, 67]. The latter observation would imply that such transformants are angiogenic [67], and, at least in the case of *RAF* transfectants, upregulation of VEGF [10] has been formally demonstrated. However, in other studies, constitutive activation of the raf/MEK pathway was insufficient for full tumorigenic transformation [68]. Furthermore, in epithelial IEC-18 cells, overexpression of a gain-of-function mutant of

*Table 2. Examples of cytokines conceivably acting on endothelial cells in an autocrine manner*

| Cytokine | Documented angiogenic activity | References |
|---|---|---|
| bFGF | Yes | [123] |
| VEGF | Yes | [124] |
| IL-1 | ? | [125] |
| IL-6 | No | [126, 127] |
| IL-8 | Yes | [128] |
| HGF | Yes | [129] |
| TGF-β | Yes | [76] |

IL, interleukin; HGF, hepatocyte growth factor; TGF, transforming growth factor.

MEK-1 was insufficient to upregulate VEGF to the level observed in ras transfectants (Mitsuhashi, Rak, Ahn and Kerbel, Sunnybrook Health Science Center, Toronto, Canada, and University of Colorado, Boulder, CO, U.S.A.). Therefore, alternative signals may be required for *RAS*-dependent VEGF expression, possibly involving elements of the rho/rac-1/cdc-42 cascade of small GTP binding proteins.

Paracrine production of angiogenic polypeptides by oncogenically transformed cells most probably represents a small, albeit significant portion of the overall regulation of angiogenesis. While various efferent signals received by microvascular endothelial cells trigger a series of events leading to blood vessel formation, at the same time endothelial cells may send afferent, paracrine signals, which in turn could affect the behaviour of juxtaposed stromal and tumour cells.

## PARACRINE EFFECTS OF ENDOTHELIAL CELLS ON TUMOUR CELL BEHAVIOUR

*Endothelial-derived paracrine signals*

Like tumour cells, endothelial cells are a rich source of bioactive substances which may act in an endocrine, paracrine, juxtacrine or autocrine manner [69, 70]. An interesting corollary to this point is that a number of putative angiogenesis factors have been shown to be produced by endothelial cells themselves either *in vivo* or under various experimental conditions (Table 2) and may contribute to endothelial cell function and survival [71]. While this is clearly an attractive possibility, endothelial cells in culture, and most likely *in vivo*, depend on exogenous growth factors both in quiescence and upon onset of angiogenesis.

Among the numerous bioactive substances produced by endothelial cells, many possess the potential to alter the behaviour of various types of adjacent cells (Table 3). One of the more notable, recent examples of this kind of an interaction was described in the study by Yamane and associates, dealing with reciprocal communication between endothelial cells and hepatocytes in the regenerating liver [72]. In this study, hepatocytes were shown to be the major source of VEGF, upon which hepatic sinusoidal endothelial cells are entirely dependent for their growth and survival. Conversely, hepatocyte growth factor/scatter factor (HGF), which is required for liver regeneration, was produced mainly by endothelial cells, and its receptor, c-met tyrosine kinase, was expressed primarily by hepatocytes. Upon liver injury, endothelial cells were shown to upregulate the expression of both HGF ligand and the VEGF receptor (flt-1) allowing a more efficient signal exchange with hepatocytes. Presumably, such endothelial–hepatocyte cross-talk and co-

*Table 3. Bioactive products of endothelial cells which could affect the behaviour of adjacent tumour cells*

| Factor | Abbreviation | Reference |
|---|---|---|
| Platelet derived growth factor | PDGF | [130] |
| Connective tissue growth factor | CTGF | [131] |
| Basic fibroblast growth factor | bFGF | [123] |
| Endothelial derived growth factor | EDGF | [130, 132] |
| Heparin-like inhibitor | | [133] |
| Hepatocyte growth factor | HGF | [72] |
| Transforming growth factor β | TGF-β | [76] |
| Insulin-like growth factor 1 | IGF-1 | [134] |
| Insulin-like growth factor 2 | IGF-2 | [41] |
| Heparin-binding epidermal growth factor | HB-EGF | [135] |
| Burst promoting factor | | [136] |
| Interleukin-1 | IL-1 | [137] |
| Interleukin-6 | IL-6 | [91] |
| Interleukin-7 | IL-7 | [138] |
| Interleukin-8 | IL-8 | [139] |
| Interleukin-9 | IL-9 | [80] |
| Leukaemia inhibitory factor | LIF | [140] |
| Granulocyte colony stimulating factor | G-CSF | [141] |
| Macrophage colony stimulating factor | M-CSF | [142] |
| Granulocyte-macrophage colony stimulating factor | GM-CSF | [143] |
| Steel factor | SLF | [144] |
| Melanoma growth stimulatory activity | MGSA | [145] |
| Macrophage chemotactic peptide | MCP | [146] |
| Transferrin | | [147] |
| Endothelin-1 | ET-1 | [75] |
| C3b complement fragment | C3b | [148] |

*Table 4. The effect of cytokine treatment on growth and VEGF expression of human tumour cell lines*

| Cell line | Origin | Treatment | Growth response | VEGF mRNA |
|---|---|---|---|---|
| WM35 | Human melanoma | IL-6 (20 ng/ml) | Inhibition | No effect |
| WM902b | Human melanoma | IL-6 (20 ng/ml) | Inhibition | Mild stimulation |
| MeWo | Human melanoma | IL-6 (20 ng/ml) | No effect | No effect |
| WM9 | Human melanoma | IL-6 (20 ng/ml) | No effect | No effect |
| | | TGFβ (2 ng/ml) | Inhibition | Stimulation |
| MCF7 | Human breast carcinoma | IL-6 (20 ng/ml) | Inhibition | Stimulation |
| ACHN | Human renal cell carcinoma | IL-6 (20 ng/ml) | No effect | No effect |

ordinated gene expression is essential for proper assembly of the tissue repair reaction. This scenario can be extrapolated to conditions found in cancer. For example, human melanoma cells which are known to be responsive to treatment with HGF [73], also produce various amounts of VEGF [74] (Rak, Florenes and Kerbel, Sunnybrook Health Science Center, Ontario, Canada). Such cells frequently metastasise to the liver (a preferential site of metastasis, in the case of ocular melanoma).

Another interesting example of endothelial-derived paracrine activity is the effect on fibroblasts described recently by Villaschi and Nicosia in relation to their *in vitro* model of granulation tissue formation [75]. In this endothelial–fibroblast coculture system, fibroblasts were able to enhance endothelial cell survival, angiogenesis and collagen gel contracting capacity. However, the presence of endothelial cells and particularly their endothelin-1 (ET-1) protein product, led to increased expression of α-smooth muscle actin, contractile properties and overall myofibroblastic transformation of fibroblasts. The authors of this study pointed out that experiments with breast cancer associated fibroblasts demonstrated that another endothelial derived molecular mediator, TGF-β, is capable of inducing a similar myofibroblastic transformation. TGF-β is produced by a host of different cell types (including various types of endothelium) and is known to be released from the cells in a latent form which requires proteolytic cleavage for unmasking its activity. Such spontaneous TGF-β activation was observed in endothelial–fibroblast co-culture experiments and is thought to depend on cell–cell contact [76]. It is tempting to speculate that a similar mode of TGF-β activation may operate in tumours. Tumour–endothelial interactions, with or without enzymatic co-operation with stromal fibroblasts, may result in release of activated TGF-β into the tumour microenvironment resulting in a direct impact of this cytokine on such important biological variables as tumour cell proliferation [77], ECM composition and angiogenesis [78]. Several other interesting examples of paracrine endothelial influence on fibroblasts and smooth muscle cells have also been described, and similar mechanisms may be applicable to tumour biology [43, 70, 79]. The available space and focus of this article does not permit a more extensive discussion of these findings or review of the extensive literature on adhesive, cytotoxic and enzymatic cell–cell interactions involving endothelium [70, 80–83].

*Changes in tumour cell behaviour induced by endothelial cells*

There is a relatively small number of studies pointing directly to the possible paracrine influence of endothelial cells on tumour cell growth and metastasis. Recently,

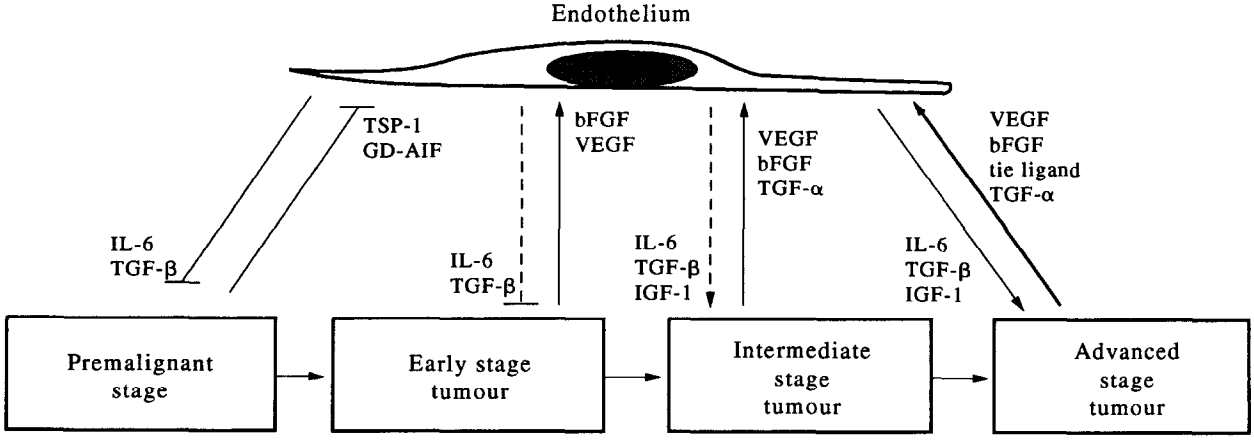


**Figure 4. Reciprocal interactions between tumour cells and endothelial cells during tumour progression. The growth of normal and premalignant cells is frequently inhibited by cytokines such as IL-6, IL-1 and TGF-β which are known to be secreted by endothelial cells. Premalignant and early stage tumour cells tend to be non-angiogenic and frequently express angiogenesis inhibitors, such as TSP-1 and GD-AIF. With disease progression, the sensitivity of tumour cells to growth inhibition by endothelial-derived cytokines (and other factors) is reduced, lost or 'switched' to growth stimulation. Additional growth factors may be produced by activated endothelial cells, which can stimulate proliferation, migration, survival and angiogenic properties of more malignant tumour cells. At the later (advanced) stages of tumour progression, the balance of angiogenesis stimulator and inhibitors produced by tumour parenchyma favours both blood vessel formation and tumour cell growth. Thus, the mutual inhibition of endothelial and parenchyma cells is converted over time to a reciprocal stimulation pattern. The diagram is based on a compilation and extrapolation of data from different systems (mainly melanoma and colorectal carcinoma; compare text and Table 3) and does not literally represent the events in a particular type of tumour.**

Hamada and associates [84, 85] have documented that liver sinusoidal endothelial cells produce growth and motility factors for metastatic variants of murine large cell lymphoma RAW117. One of these migration stimulating factors was subsequently identified as the C3b fragment of complement [85]. The inhibitory effect of sinusoidal endothelium on liver metastasis formation by another mouse lymphoma cell line has also been recently described [13].

Nicosia and colleagues have shown, in two different models of *in vitro* angiogenesis (the rat aorta model and endothelial spheroid model), that growth and invasion of the rat bladder squamous carcinoma cell line NBT-II-81 was accelerated by the presence of microvascular sprouts [86, 87]. These sprouts were gradually replaced by cords of invading tumour cells in which endothelial cells were dislodged from their matrix and degenerated over time (by apoptosis?). Importantly, in this *in vitro* model system, endothelial channels were not perfused and did not contain blood; hence, nutrient and oxygen gradients could not have accounted for the induction of a more aggressive tumour cell behaviour. The authors concluded that it was the endothelial cell derived ECM which played a causative role in this case [87]. While the ECM may indeed alter growth, motility and survival of various cell types [88], it also may retain soluble mitogenic or mitogenic cytokines, the presence of which was not tested in this model system.

In a more recent study, SCID mice were grafted with human skin fragments, and subsequently the successful implants were injected with human breast cancer cells. After tumours arose and became vascularised (by human blood vessels from the implants), the mice were injected with a neutralising antibody (LM609) against the $\alpha_v\beta_3$ integrin. This antibody is known to bind to sprouting endothelial cells and to possess anti-angiogenic activity [89]. As expected, the treatment had an anti-angiogenic and anti-tumour effect [90]. However, it turned out that injections of LM609 antibody also greatly reduced the invasiveness of tumour cells themselves. This was clearly an unexpected and indirect effect since tumour cells used in this study did not express the $\alpha_v\beta_3$ receptor. The authors hypothesised that a less invasive behaviour of tumour cells in the absence of angiogenic endothelial cells is due to secretion by the latter of large quantities of proteolytic enzymes into the tumour microenvironment. An alternative explanation was that leakage of growth factors from serum into well-perfused angiogenic tumour parenchyma could cause enhanced invasiveness of tumour cells [90]. The latter possibility seems less likely in light of the aforementioned *in vitro* studies of Nicosia [87]. While modulation of endothelial-dependent proteolysis clearly could be involved in this case, the production of growth factors by endothelial cells themselves and their paracrine effect of tumour cell growth and motility is worth considering as well. Interleukin 6 (IL-6) could be one of the possible mediators of such a direct interaction. This cytokine is produced by endothelial cells *in vitro* [91] and *in vivo* [92], and is known to stimulate the motility of certain breast cancer cell lines in culture [93]. IL-6 is also a survival factor for tumour cells such as multiple myeloma [94]. Interestingly, C6 rat glioma or A431 human carcinoma, when treated with high concentrations of IL-6, can upregulate expression of VEGF [95]. Since certain angiogenic factors, including bFGF [96] and VEGF (Rak, Sunnybrook Health Science Center, Ontario, Canada) may, in turn, stimulate production of IL-6 by endothelial cells, there is a potential for a pro-angiogenic paracrine positive feedback loop involving both tumour cells and endothelium. It is puzzling that some highly malignant cell lines maintain residual sensitivity to growth inhibitory effect of IL-6 ([97], Table 4) or TGF-β (WM9 metastatic human melanoma, [98], Table 4). One possible explanation for why such cells have not been selected against might be that the direct anti-proliferative effect of these cytokines is outweighed *in vivo* by an indirect stimulation of angiogenesis through TGF-β (or IL-6)-dependent upregulation of VEGF (Table 4). However, our preliminary results indicate that overexpression of VEGF upon IL-6 (and probably TGF-β) treatment may not be a universal feature among human tumour cell lines (compare with Table 4).

The paracrine effect of endothelial cells on tumour cell behaviour may change with tumour progression. Perhaps this may be best appreciated in angiogenic tumours which originate from avascular tissues. The latter is certainly a common situation in the case of human cutaneous melanoma where the cell of origin, normal melanocyte, usually resides in the epidermis with no direct contact with dermal blood vessels. During initial stages of melanoma development, particularly the so called radial growth phase (RGP) and 'thin' vertical growth phase (VGP), lesions tend to remain avascular, slow growing and for that matter, are in most cases surgically curable (i.e. metastatically incompetent) [99, 100]. Normal melanocytes, as well as cells derived from the early stage melanoma, are exquisitely sensitive to growth inhibition induced by a number of inflammatory and mesenchymal cytokines including IL-6, IL-1, tumour necrosis factor alpha (TNF-α) and TGF-β [98, 101, 102]. This may imply that, at the outset of the process, some of these factors produced by fibroblasts, inflammatory cells and endothelium in the dermis may contribute to spatial restriction of tumour growth to the epidermis. In contrast, cell lines derived from later stages of melanoma progression are not responsive to the growth inhibitory effects of these cytokines i.e. display a 'multicytokine resistance' phenotype [103]. There is also some evidence to suggest that progression of other types of solid tumours may be associated with increasing resistance to growth inhibitory cytokines. In the previously mentioned IEC-18 system, conditional expression of mutant *H-RAS* reduced the sensitivity of epithelial cells to growth inhibition by TGF-β [104].

From both the prognostic and pathobiological point of view, the turning point in melanoma progression seems to be the transition from a 'thin' intra-epidermal, avascular lesion to a 'thick' (>0.76 mm) VGP melanoma. This transition is not only associated with tumour cell invasion of, and growth within the dermal mesenchyme, but also with the onset of angiogenesis—which by itself may be both the cause and effect of the increasing tumour thickness [30, 105]. Even though the molecular basis of the 'angiogenic switch' in melanoma is not fully understood [105], it has been shown that production of potentially angiogenic polypeptides, such as bFGF, is elevated *in situ* in a proportion of early stage lesions [106]. Also, expression of various amounts of VEGF were detected in such melanoma specimens (Florenes, Meladsmo, Rak, Kerbel and Fodstad, Sunnybrook Health Science Center, Ontario, Canada and

Norwegian Radium Hospital, Oslo, Norway). In fact, the WM35 melanoma cell line, which has been derived from an early (RGP) lesion, is capable of producing measurable quantities of VEGF in culture (Rak, Bani, Yeo, Dvorall and Kerbel, Sunnybrook Health Science Center, Ontario, Canada and Beth Israel Hospital, Boston, Massachusetts, U.S.A.). One might ask then, what are the consequences of the early onset of angiogenesis in melanoma when cytokine sensitive tumour cells are confronted with cytokine producing endothelium? In direct co-culture experiments such confrontation reproducibly leads to growth inhibition of early stage melanoma cells, and this is partially abrogated by addition of the anti-IL-6 neutralising antibody [107]. Moreover, in a clinical study concerned with the prognostic significance of tumour vascularity in human melanoma, Barnhill and colleagues made a similar, albeit seemingly paradoxical, observation [108]. In that study, the subpopulation of 'thin' (<1 mm) melanoma lesions with signs of histological regression actually showed an increase in blood vessel counts compared to samples without regression. It is not entirely clear what caused the regression and what might be the significance of high vascularity in such cases. The authors proposed that the antitumour inflammatory reaction may have been facilitated by angiogenesis (the reverse may also be true). However, differences in the extent of lymphocytic/inflammatory infiltration between regressing and non-regressing lesions were not analysed or apparent in this study. Furthermore, since histological regression is usually followed by rapid melanoma progression and metastasis, it is considered a negative prognostic indicator [108, 109]. However, lymphocytic infiltrates are usually associated with a favourable prognosis in melanoma [110]. Currently, nothing seems to suggest that a direct paracrine influence of endothelial cells on melanoma cells should be discarded in terms of explaining such observations. One may speculate, therefore, that the inhibitory effect of endothelial cell products on tumour cells may, at early stages of melanoma progression, facilitate selection of more aggressive (i.e. growth non-inhibitable) tumour cell variants [47]. However, the molecular mediators of such an interaction and their exact effect on susceptible tumour cells are far from understood. The presence of histological regression in highly vascular populations of thin melanomas may suggest that not only is inhibition of tumour cell proliferation involved, but perhaps also their death (apoptosis). Theoretically, there are at least two scenarios which may lead to this counter-intuitive outcome. Endothelial cells may release cytokines (IL-6, TGF-β), chemokines or other bioactive substances (prostaglandins, nitric oxide, reactive oxygen species) which, under certain circumstances, may not only cause growth arrest but also directly induce apoptosis of tumour cells [111, 112]. Growth of advanced melanoma cells is usually not inhibited by such factors and would be expected to show, overall, increased resistance to apoptosis. This may conceivably allow such cells not only to withstand the presence of endothelium, but also to use new blood vessels to promote tumour cell growth and survival. Another possibility is that the rapid influx of growth factors into a newly vascularised tumour may cause inappropriate cell cycle entry of not completely transformed cells, and thereby indirectly increase the rate of apoptosis. It has been shown that in some cell types coexistence of 'conflicting' growth stimulatory and growth inhibitory signals may lead to apoptosis [113, 114]. Whichever explanation is correct, the histological regression of highly vascular thin melanomas remains a puzzling and paradoxical phenomenon and, interestingly, is not an entirely isolated case.

In an elegant transgenic model of RIP-Tag mice, Hanahan's group has shown recently that directing expression of the *SV40Tag* oncogene to pancreatic islets leads to development of insulinomas progressing through a distinct angiogenic phase [32; see also Hanahan *et al.* in this Issue, p. 2386]. Prior to emergence of overt malignant tumours, angiogenic precursor lesions display a massive increase in the rate of both proliferation and apoptosis, the latter (but not the former) of which is then attenuated at later stages of progression [35, 115]. A simultaneous co-expression of *bcl-x*$_L$ and *Tag* transgenes ameliorated the pro-apoptic effect associated with the angiogenic switch, but did not accelerate the accumulation of angiogenic precursor islets. Based on this observation, the authors discarded the possibility that the stepwise development of apoptosis resistance is a causative factor in transition to the angiogenic phenotype in this model. More importantly, they also concluded that even though angiogenesis may act as one of the apoptosis reducing factors at the later stages of tumour progression, this is not the case at the stage of *Tag*-induced hyperplasia. Furthermore, they postulated that a high rate of apoptosis which accompanied the onset of angiogenesis in hyperplastic islets is most likely due to some 'microenvironmental' influence associated with blood vessel formation. There is obviously no formal proof that a paracrine effect of endothelial cells was playing a role in this 'microenvironmental' change. However, the latter possibility is testable, provided blood vessel perfusion can be ruled out as a factor. A rather elegant *in vitro* angiogenesis assay, which was previously used by Folkman and Hanahan [32] to detect the onset of angiogenesis in isolated hyperplastic islands, could be adapted for this purpose. In this assay, spheroids of pancreatic cells are co-cultured with endothelial cells and the directional movement of the latter is observed whenever an angiogenesis inducer is released into the medium. Instead of using endothelial cells as a 'read-out', one could use them as a 'treatment' i.e. evaluate the rate of proliferation and apoptosis within the pancreatic islets after they have become angiogenic in the presence of activated endothelial cells.

It is not inconceivable that the inhibitory or pro-apoptic effect of angiogenesis on premalignant lesions may extend beyond thin melanoma and the RIP-Tag model system [35]. The observation and analysis of early stages of tumour progression has always been a difficult task, as has the study of the events accompanying the onset of angiogenesis. However, in the case of the more studied, advanced stage tumours (e.g. advanced stage melanoma), angiogenesis appears to support three-dimensional growth and tumour cell survival. It is, therefore, apparent that the pattern and functional significance of tumour–endothelial cell interactions may undergo a significant change during tumour progression (Figure 4).

## CONCLUSION: THE 'TWO-WAY' INTERACTIONS AND THE 'FOURTH DIMENSION' IN TUMOUR ANGIOGENESIS

In the seventh century A.D. a Persian physician by the name of Paul of Aegina gave the following description of

cancer: *Cancer is an uneven swelling, rough, unseemly, darkish, painful, and sometimes without ulceration. [ . . . ] It has its veins stretched on all sides as the animal, the crab (cancer) has its feet, whence it derives its name.* The reference to blood vessels is very apparent. However, it is only fairly recently that the contribution of angiogenesis to solid tumour formation is beginning to be appreciated more fully in both a pathobiological and therapeutic sense. While a number of putative tumour angiogenesis factors have been identified and their biological effects characterised in detail, it is perhaps not out of place to ask what is the biological *context* and *time frame* of their production and activity. In this sense, the quantity and composition of angiogenic growth factors and inhibitors produced by tumour cells will probably change with progression of the genetic, phenotypic and microenvironmental characteristics of a given tumour. Similarly, the phenotype of endothelial cells which are recruited to the tumour site by direct or indirect-acting pro-angiogenic factors may change with the change of the inducing stimulus. In relation to the latter, it is worth drawing attention to the fact that different angiogenic growth factors can synergise or interact with each other in a number of different ways [116–118]. Predicting the outcome of such complex interactions may be quite difficult. For example, it is known that expression of KDR receptor kinase (VEGFR2) on endothelial cells can be upregulated in the presence of bFGF, while treatment with TNF-α apparently has the opposite effect. Alternatively, both bFGF and TNF-α are angiogenic and are known to upregulate VEGF in various types of cells [199]. Naturally, such diverse, combinational influences may produce an enormous potential for multistep sequential changes in the angiogenic capacity of tumour cells during the course of tumour progression. Little is known about corresponding sequential changes in the phenotype of tumour associated endothelial cells. A number of molecules have been found to be upregulated in such cells including KDR, flt-1, tie-1, tie-2 tyrosine kinases and $\alpha_v\beta_3$ integrin [47]. It is plausible that a similar change may occur in their growth factor and cytokine production profile and their pattern of paracrine interactions with adjacent tumour cells. Understanding the principles governing this complex network of interactions and its evolution during the natural history of different types of solid tumours may bear numerous diagnostic, prognostic and therapeutic opportunities.


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