Angiogenesis and apoptosis are cellular parameters of neoplastic progression in transgenic mouse models of tumorigenesis

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ABSTRACT The epidemiology and histopathology of human cancers and studies of animal models of tumorigenesis have led to a widely-accepted notion that multiple genetic and epigenetic changes have to accrue for the successful development of a malignant phenotype. Tumor growth and expansion requires an ability not only to proliferate, but also to down-modulate cell death (apoptosis) and activate angiogenesis to produce a tumor neovasculature. This review will describe the interplay between apoptosis and proliferation, as well as the characteristics of the angiogenic phenotype in two transgenic mouse models of multi-step tumorigenesis, namely, pancreatic islet cell carcinomas and squamous cell carcinomas of the skin.

KEY WORDS: growth, growth factors, hormones, gene knockout, mouse embryo

Introduction

Transgenic models for human disease

The conversion of normal cells into invasive cancers with metastatic potential is a process that involves several steps (Fearon and Vogelstein, 1990). These steps are manifested in distinguishable histological and temporal stages (Foulds, 1969; Henson and Albores-Saavedra, 1993), e.g., normal tissue, hyperplasia with a high incidence of proliferating cells, dysplasia with the induction of angiogenesis, solid tumors, and finally metastases. Analysis of the later stages of tumor progression has resulted in a multi-step theory of tumorigenesis based on genetic changes involving activation of oncogenes, inactivation of tumor suppressor genes, and altered expression of cancer-associated molecules (Knudson, 1986; Klein, 1987; Fearon and Vogelstein, 1990). However, the early stages of tumorigenesis have remained largely inaccessible and thus, the critical determinants undefined.

A powerful system to investigate tumorigenesis has resulted from the ability to manipulate the mouse germ line through the introduction of new genetic information (Hanahan, 1989) and through the targeted disruption of existing genes by homologous recombination (Capemchi, 1988; Zimmer, 1992). Lines of transgenic mice have been produced with a heritable predisposition to develop specific cancers, as a result of the expression of oncogenes or the abrogation of tumor suppressor genes (Hanahan, 1988; Adams and Cory, 1991; Clarke et al., 1992; Donehower et al., 1992; Jacks et al., 1992; Lee et al., 1992). These mice recapitulate the ontogeny of tumorigenesis, often through a series of reproducible temporal and histological stages that are accessible to molecular analysis.

Our laboratory has been successful in developing and manipulating transgenic mouse models that have provided valuable clues into the multi-step nature of tumorigenesis. Two of these models will be discussed and compared in this review. The first model of tumorigenesis employs the rat insulin II gene regulatory region (RIP) to target expression of the Simian Virus-40 (SV40) large Tumor antigen (Tag) oncoprotein to the β cells of the pancreatic islets (Hanahan, 1985). These mice develop insulinomas and islet cell carcinomas (Fig. 1). The second model is produced by expressing the early region genes, including the E6 and E7 oncogenes of human papillomavirus type 16 (HPV-16) under the control of the human keratin 14 (K14) promoter/enhancer (Arbeit et al., 1994;
Coussens et al., 1996). These mice develop varying grades of squamous cell carcinoma (Fig. 1). This review will focus on various features of tumorigenic progression common to both transgenic models, e.g., multi-stage progression, cellular proliferation, apoptosis, and onset of angiogenesis prior to overt malignancy, as well as the parameters unique to each model, such as terminal differentiation, constitutive expression versus incremental upregulation of heparin binding growth factors, and variations in the microenvironment specific to each neoplastic site.

**β-cell islet carcinogenesis**

One of the first transgenic animal models of human malignancy was the RIP1-Tag mouse model of islet cell carcinogenesis, of which the RIP1-Tag2 line has been the prototype (Hanahan, 1985). In this model, due to the control of the rat insulin II promoter, expression of the SV-40 large T-antigen is restricted to the insulin-producing β cells of the islets of Langerhans in the pancreas. The Tag oncoprotein possesses a number of functional activities, including the ability to bind and thereby inactivate two tumor suppressor proteins, pRB and p53 (Ludlow, 1993). The expression of Tag in Rip1-Tag2 mice is concomitant with the onset of insulin expression at embryonic day 9 in the pancreatic diverticulum and persists in the β cells of all islets through adulthood (Alpert et al., 1988). Despite the embryonic onset of Tag expression, consequences are not apparent until mice are approximately 4-6 weeks old. Beginning at this age, individual islets switch from a low proliferation index (ca. 3% of the β cells in S-phase) to a high proliferation index (15-20% of the β cells in S-phase) as measured by 5-bromo-2′-deoxyuridine (BrdU) incorporation (Fig. 2A; Teitelmann et al., 1988; Naik et al., 1996). The proliferating β cells are diffusely scattered throughout each islet, rather then being localized in a focal sector, which may suggest the involvement of a diffusible growth factor in the onset of hyperproliferation. By 6-8 weeks, a subset of hyperplastic islets acquire an angiogenic phenotype, histologically detectable by increased blood vessel density associated with the appearance of hemorrhages (Figs. 1B and 3). Moreover, angiogenic islets, in contrast to normal and hyperplastic islets, secrete soluble angiogenic factors that are chemoattractive and mitogenic for endothelial cells (Folkman et al., 1989; G. Bergers unpublished observation). Finally by 12-14 weeks of age, approximately 20% of angiogenic islets develop into highly vascularized solid islet tumors that can differ in their invasive capacity (Fig. 1). Although most of the tumors remain encapsulated (Fig. 1C), 1-2% can
become invasive by down-regulating the cell adhesion molecule E-cadherin (Fig. 1D; Perl et al., 1998).

**Squamous epithelial carcinogenesis**

Transgenic mice expressing the HPV16 early region genes, including the E6/E7 oncogenes, under the control of the human keratin-14 promoter/enhancer, develop reproducible multi-stage progression to invasive squamous cell carcinoma (SCC) of the epidermis (Arbeit et al., 1994; Coussens et al., 1996). In K14-HPV16 mice, skin keratinocytes faithfully recapitulate the hallmark stages of SCC development in humans (Fig. 1E-H). By one month of age, hyperplastic lesions are detectable throughout ear epidermis and by three months of age similar lesions are focally present on truncal skin. These areas of hyperproliferative activity involve all four epidermal cell layers, resulting in a few-fold increase in thickness of each layer. Between 3 and 6 months of age, focal areas of dysplasia can be found on both ear and truncal skin. Such areas represent premalignant lesions that are 100% penetrant and, depending on the genetic background, are predisposed to progress into varying histologic grades of SCC by 7 to 12 months of age (Coussens et al., 1996). Premalignant ear lesions typically give rise to Well-Differentiated Squamous cell Carcinomas (WDSC), whereas truncal dysplastic lesions more commonly give rise to Moderate-Poorly Differentiated Squamous cell Carcinomas (M-PDSC).

**Proliferation versus programmed cell death**

Cumulative tumor growth results not only from increased cellular proliferation but also from the ability of cancer cells to circumvent the normal pathways leading to apoptosis (or programmed cell death, PCD) (White, 1996). Apoptosis is a genetically determined cellular program which normally protects the organism from aberrant proliferation, maintains tissue size, and eliminates mutant or diseased cells which represent a threat to tissue homeostasis (Kerr et al., 1987; Raff, 1992). “Classical” apoptotic cell death is characterized by distinctive morphological changes in the dying cell, including shrinkage, loss of cell to cell contact, chromatin condensation, and internucleosomal degradation of DNA (Wyllie, 1980). In addition, programmed cell death may also occur in alternative forms that are not necessarily defined by the parameters described above, for example during terminal differentiation of keratinocytes and colonic epithelial cells, or during cellular necrosis of various tissues (Ellis et al., 1991; Ucker, 1992; White, 1996; Stern et al., 1997). Thus, it seems likely that tumors might take advantage of diverse mechanisms to gain resistance to “classical” and “non-classical” cell death programs.

**Apoptosis in Tag+ islets**

Our laboratory has investigated the patterns, regulation and importance of apoptosis in the development of islet cell carcinomas in Rip-Tag mice. We have shown that the rate of apoptosis rises incrementally in the premalignant stages of islet cell carcinogenesis, and then drops in the frankly malignant form (Fig. 2B; Naik et al., 1996). Moreover, our analyses collectively implicate apoptosis as a critical regulator of tumorigenesis and tumor growth (Christofori et al., 1996; Naik et al., 1996; Parangi et al., 1996). The Tag oncoprotein functionally inactivates the p53 tumor suppressor,
which has previously been demonstrated to be a major effector of apoptosis in a number of cancer models (White, 1996). Indeed, we have shown, by crossing Rip-Tag transgenic into a p53 null background, that the tumor phenotype and patterns of apoptosis are comparable in Rip-Tag, p53-/- and Rip-Tag, p53+/+ mice (Naik et al., 1996), consistent with abrogation of p53 function by the Tag oncprotein. Although p53 is clearly an important regulator of apoptosis, it is increasingly clear that p53-independent apoptotic pathways exist in many cell types. In particular, apoptosis is a major factor in the islet cell carcinogenesis, despite the absence of p53 function. Resistance towards apoptosis involves three identified mechanisms: activation of the survival factor IGF-2 (Naik et al., 1994, 1996), upregulation of bcl-XL gene (Naik et al., 1996), and ongoing angiogenesis (Parangi et al., 1996). Several lines of evidence suggest that a reduction in the rate of apoptosis is critical to the formation and subsequent growth of solid tumors: i) if IGF-2 function is abrogated in Rip-Tag mice by crossing them into a IGF-2/null background, smaller tumors with increased apoptosis arise; ii) if bcl-XL is ectopically upregulated in RIP-Tag mice by crossing them with RIP-bcl-XL transgenic mice, apoptosis is reduced in the progenitor stages, and the incidence of progression to solid tumors is increased; iii) if angiogenesis is impaired with angiogenesis inhibitor compounds, smaller tumors with increased apoptosis result (Parangi et al., 1996; G. Bergers unpublished observation). It is important to emphasize that in all three cases the proliferative index (fraction of cells in S-phase) does not change substantially, suggesting that tumor size and incidence is associated with down-regulation of apoptosis but not up-regulation of proliferation during progression from progenitor stages. Thus, the rate of apoptosis is a critical factor in the pathway to islet cell carcinoma.

**Apoptosis in E6/E7+ keratinocytes**

Normal stratified epidermis is a dynamic tissue which undergoes constant renewal and exhibits a highly coordinated program including proliferation and terminal differentiation. In K14-HPV16 transgenic mice, hyperplastic and dysplastic lesions are characterized by a progressively increased proliferative index that reaches a plateau at the dysplastic stage prior to solid tumor formation (Fig. 2C; Arbeiter et al., 1994; Coussens et al., 1996). Accompanying this increased proliferation is an alteration in the repertoire of keratin intermediate filament proteins correlating with loss of terminally differentiating keratinocytes (Coussens et al., 1996). It is clear that while enhanced proliferation and loss of terminal differentiation are necessary in the earlier stages of carcinogenesis, they are insuf-

**TABLE 1**

<table>
<thead>
<tr>
<th>Islet Carcinoma Model</th>
<th>Normal</th>
<th>Hyperplastic</th>
<th>Angiogenic</th>
<th>Tumor</th>
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<tbody>
<tr>
<td>aFGF</td>
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<td>bFGF</td>
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<tr>
<td>VEGF-A</td>
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<tr>
<td>VEGFR-1 (flt-1)</td>
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<td>++</td>
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<tr>
<td>VEGFR-2 (flk-1)</td>
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<tr>
<th>Squamous Carcinoma Model</th>
<th>aFGF (FGF-1)</th>
<th>bFGF (FGF-2)</th>
<th>VEGF-A</th>
<th>FGFR-1</th>
<th>FGFR-2</th>
<th>VEGFR-2 (flk-1)</th>
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<td>Hyperplastic</td>
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<tr>
<td>Angiogenic</td>
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<td>Tumor</td>
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Parts of the data shown have been reported elsewhere (Christofori et al., 1995; Arbeiter et al., 1996; Hanahan et al., 1996; Smith-McCune et al., 1996). FGFR-1 and FGFR-2 expression in the islet cell model is undetectable by in situ hybridization analysis (Olson, D., unpublished observations). (+++) and (+) are meant to imply relative expression levels as observed by in situ hybridization analysis. 1 Elevated expression was observed in vessels surrounding WDSC’s as well as in neoplastic cells of M-PDSC’s but was absent from neoplastic cells of WDSC’s.
cient to induce malignant conversion, as all transgenic mice exhibit focal areas of the highly proliferative precursor dysplastic lesions, yet only 40-50% develop SCC.

Griep and colleagues have studied the roles of the two HPV16 oncoproteins, E6 and E7, in lens epithelia and have revealed that the E7 oncoprotein simultaneously induces cell proliferation while concomitantly inhibiting morphological differentiation of epithelium. The ultimate fate of E7-expressing cells in the lens is ‘classical’ apoptotic death which can be inhibited by the E6 oncoprotein (Pan and Griep, 1994, 1995). By analogy, in K14-HPV16 transgenic mice, the dynamic roles of E6 and E7 oncoproteins may serve to induce proliferation in neoplastic keratinocytes while simultaneously limiting the extent of cell death and terminal differentiation. This and other laboratories (C. Thompson, S. Yuspa, G. Evan, personal communications; L.M. Coussens, unpublished observation) have failed to reliably detect apoptosing keratinocytes in murine skin by the TUNEL method, which visualizes DNA fragmentation of apoptotic cells (Surh and Sprent, 1994). However, by examining toluidine blue stained thin (1 µm) histologic sections of transgenic skin embedded in EPON 812, morphological apoptosis (cytoplasmic condensation and nuclear pyknosis) can be detected (Figs. 1G and 2D). The fact that the TUNEL method did not detect DNA fragmentation in transgenic skin suggests that neoplastic keratinocytes undergo an alternative pathway that does not utilize DNAse activation. This preliminary analysis suggests that keratinocytes exhibit enhanced proliferation and the gradual loss of capacity for terminal differentiation, induction of a “non-classical” apoptotic program increases in premalignant neoplastic cells (Fig. 2D). Following malignant conversion, however, the proliferative index of neoplastic cells remains high (Fig. 2C), whereas the proportion of cells undergoing pyknotic death declines (Fig. 2D). We are now beginning to dissect the regulators of cell death in this squamous epithelial model and, given the data reported here, other genetic factors controlling apoptosis, in addition to the HPV16 oncoproteins and p53, will be considered.

The angiogenic switch occurs prior to solid tumor formation

Formation of new blood vessels from existing vessels (angiogenesis) is a central event in normal tissue development and in tissue remodeling, e.g., inflammation, wound healing, neoplasia, etc. (Hanahan and Folkman, 1996). During angiogenesis, endothelial cells, stimulated by angiogenic molecules, change their genetic program. They re-enter the cell cycle, produce proteolytic enzymes, migrate, and then differentiate again to form functional new blood vessels. Tumor cells are able to gain access to the host vasculature by expressing a variety of angiogenic factors in order to procure nutrients and oxygen. Indeed, without the ability to recruit new blood vessels, most solid tumors would not grow beyond 1-2 mm in diameter and fail to metastasize (Folkman and Hanahan, 1991). Therefore, induction of angiogenesis permits rapid tumor expansion and also increases the risk of metastasis via intravasation into the vasculature. In both the Rip-Tag and K14-HPV16 transgenic models of tumorigenesis, extensive vascularization and ongoing angiogenesis has been observed not only in the end-stage tumors but also in the dysplastic tissue. This suggests that the angiogenic switch is a discrete event occurring prior to tumor development.

The angiogenic switch in Rip-Tag mice

The islets of Langerhans are naturally well vascularized (Fig. 3A,D). Capillaries within islets are fenestrated, facilitating exchange of islet hormones and other molecules between the endocrine cells and the blood. The islet vasculature remains quiescent in the adult animal. During neoplastic progression in Rip-Tag transgenic mice, an ‘angiogenic switch’ can be demonstrated by 6-8 weeks of age, in which over a few week period, approximately 20% of the hyperplastic islets evidence neovascularization (Figs. 1B and 3B). Only those islets that acquire the angiogenic phenotype have the potential to further develop into tumors. Capillaries

Fig. 5. ‘Classical’ and Non-Classical’ mechanisms involved in the angiogenic switch. In both the K14-HPV16 and Rip-Tag transgenic models of tumorigenesis, the activation of the vasculature from a state of quiescence occurs prior to solid tumor development. In the skin, quiescent vessels are normally found deep within the dermal stroma. However, following activation of the angiogenic switch, vessels with altered morphology are found tightly juxtaposed to the skin basement membrane. In contrast, β cells within the islets of Langerhans are naturally surrounded by capillaries and an arteriole. Following activation of the angiogenic switch in Rip-Tag transgenics, the proximal nature of vessels does not change, rather, the vasculature becomes more complex with additional vessel sprouting.
in the angiogenic islets are irregularly shaped, tortuous and enlarged (Fig. 3D). In addition, islet vessels become fragile and leaky, as indicated by the high number of hemorrhages present within tumors (Fig. 3C). The induction of angiogenesis has been substantiated using an in vitro bioassay for angiogenesis, involving coculture with bovine capillary endothelial cells and islets in three-dimensional collagen gels (Folkman et al., 1989). Endothelial cells remain randomly distributed around normal and hyperplastic islets, whereas angiogenic islets and islet tumors induce a dramatic response with radial alignment, proliferation and migration of endothelial cells towards the islets. These data suggest that angiogenic islets, but not hyperplastic islets, secrete angiogenic factors. Surprisingly, despite the discrete angiogenic switch, the major angiogenic factors VEGF and aFGF are expressed at high levels in normal pancreatic islets as well as in tumor islets (Christofori et al., 1995, 1996). Moreover, the VEGF receptor genes, flt and flk, are also constitutively expressed during all stages (Table 1). Thus, the absence of modulation of gene expression for two potent angiogenesis inducers and the VEGF receptors suggest that other molecules or alternative mechanisms are necessary to elicit the angiogenic switch in this model.

The angiogenic switch in K14-HPV16 mice

Skin vasculature is typically quiescent and found deep within the dermal stroma (Detmar, 1996; Fig. 4A). In K14-HPV16 transgenic mice, neoplastic progression involves an early increase in capillary density in every mouse exhibiting focal dysplastic lesions (Fig. 4). As this phenotype is fully penetrant, rapid onset of angiogenesis early in neoplastic progression is insufficient by itself to induce malignant conversion. Immunostaining for the endothelial marker CD31 reveals a perceptible increase in dermal capillary density that is first apparent in the hyperplastic stage of one month old transgenic mice (Fig. 4B). There is a striking increase in both the number and distribution of dermal capillaries in the early and advanced dysplastic lesions; numerous vessels become tightly juxtaposed to the basement membrane separating dysplastic keratinocytes from the underlying stroma (Fig. 4C). By perfusing vessels with a lectin derivative (Thurston et al., 1996), the enlarged size and chaotic arrangement of the capillaries, as well as their chemotactic attraction towards the basement membrane can be visualized (Fig. 4D). This vessel pattern is indicative of an angiogenic switch from vascular quiescence to an initial condition of modest neovascularization in early low grade lesions, followed by a striking upregulation of angiogenesis in high-grade neoplasms. Progression from the hyperplastic to the dysplastic/angiogenic stage is accompanied by up-regulation of several angiogenic factors (summarized in Table 1; Arbeit et al., 1996; Smith-McCune et al., 1996). In normal epidermis neither aFGF nor VEGF RNA levels are detectable, and in early hyperplastic epidermis the factors are barely detectable. However, a striking increase in aFGF and VEGF transcription is observed in high-grade dysplastic lesions with a further up-regulation of VEGF in the frankly malignant stage. In contrast, bFGF appears to be expressed in normal and hyperplastic epidermis, and only moderately upregulated in the dysplastic/angiogenic stage as well as in the WDSCs. Transcription of one FGF receptor, FGFR-1, is not detectable in normal and hyperplastic skin, moderately induced in the angiogenic stage and highly elevated in the M/PDSCs. FGFR-2 and VEGFR2/flk transcripts are expressed in normal epidermis and persist at high levels in all stages. Taken together, increasing expression of the angiogenic inducers aFGF, VEGF, and bFGF with malignant progression implicate a role of these molecules in the onset and progressive nature of angiogenesis in the skin.

The angiogenic switch: altered balance of inhibitors and activators

Angiogenesis can be regulated both by inducers as well as by inhibitors of endothelial cell proliferation, migration and tube formation (Folkman and Hanahan, 1991; Iruela-Arispe and Dvorak, 1997). Although the expression pattern of angiogenic factors can vary in normal tissues depending on the microenvironment, it is the dynamic balance of inducer and inhibitor activity that finally seems to determine whether an endothelial cell will remain quiescent or angiogenic. Thus, down-regulation of inhibitors and/or upregulation of inducers can tip the balance towards angiogenesis. Alternative mechanisms have also been proposed, in which angiogenic molecules are converted from inactive to active forms without an alteration in expression levels per se of that respective factor. For example, aFGF and bFGF can be activated by selective export by a number of tumor cell lines but not by normal cells (Kandel et al., 1991; Christofori et al., 1996). Furthermore, specific isoforms of sequestered VEGF and bFGF can be released from the extracellular matrix and so be activated (Brown et al., 1997; Friedl et al., 1997; Pollarak et al., 1997). Our studies of premalignant stages of tumorigenesis have uncovered two distinct expression patterns of angiogenic regulators; K14-HPV16 skin carcinogenesis is consistent with the model that upregulation of angiogenic inducer gene expression facilitates activation of the angiogenic switch (Fig. 5). In contrast, the Rip-Tag model reveals constitutive expression of angiogenesis inducer genes during both vascular quiescence and angiogenesis, implicating alternative mechanisms for activating the angiogenic switch (Fig. 5). It seems likely, given the different physiological characteristics of different tissues, that each tissue will resort to a different strategy for increasing activator or reducing inhibitor levels, ultimately leading to activation of the angiogenic switch. Thus, tissues in which the vasculature is embedded in the stroma and well separated from the epithelium, as in the skin, may utilize mechanisms that increase normally low inducer levels to elicit angiogenesis (Fig. 5). It has also previously been documented that angiogenic factors like bFGF can be stored in a sequestered form in the basement membrane and then released to partake in angiogenesis (Vlodavsky et al., 1996). In contrast, intimately vascularized organs such as the pancreatic islets (and lung, liver etc.) may utilize negative regulators to maintain a quiescent endothelium (Fig. 5). Down-regulation of inhibitor production or additional mechanisms such as regulated export or release of sequestered angiogenic factors may be involved in activating the angiogenic switch.

Conclusions

Although much has been learned by studying these two transgenic models, we expect further insights into the mechanisms of tumor progression. The multi-step nature of Rip-Tag and K14-HPV16 pathways indicates that expression of the oncoproteins T antigen and E6/E7 are insufficient in themselves to elicit the development of solid tumors, as only a small portion of oncogene-positive cells become cancers. Rather, additional genetic and
epigenetic changes need to occur, in conjunction with oncogene expression, to facilitate complete tumorigenesis. In both models, increased proliferation of neoplastic cells plateaus prior to the stage of frank malignancy, whereas apoptosis of neoplastic cells, initially enhanced in premalignant stages, clearly declines as neoplastic cells undergo malignant conversion. Thus, the ratio between proliferating and apoptosing cells appears important in determining tumor mass; tumor cells apparently acquire resistance towards apoptosis while maintaining a relatively high proliferative index. It is notable that in normal skin, proliferation and terminal differentiation are tightly coupled in order to maintain normal architecture in a continually renewing tissue. In contrast, the endocrine islets have a very low turnover and therefore contain hardly any apoptotic cells. The role of apoptosis in skin is not well understood, but it has been suggested that terminal differentiation may be a special case of apoptosis (Stern et al., 1997). Thus, apoptosis may occur in different forms, which would also explain why we did not observe TUNEL positive cells in the K14-HPV16 model although pyknotic nuclei were clearly visible. Therefore, mechanisms that lead to the onset of apoptosis in both models may be dependent on the cell type, the microenvironment as well as the turnover rate of the respective tissues. In addition, we have shown that angiogenesis is also an important component of tumor progression. In Rip-Tag mice, if angiogenesis is impeded by either limited availability of survival factors, e.g., IGF-2, or, treatment with angiogenic inhibitor compounds, relative tumor size is reduced while the apoptotic index in neoplastic cells increases. Although such experiments have yet to be performed in the squamous carcinogenesis model, we expect solid tumor formation or growth will also be impeded by inhibiting angiogenesis. In summary, two key events, down-modulation of apoptosis and onset of angiogenesis, are important contributors to tumor progression. The mechanisms, however, that are responsible for these events can differ between tissue types, i.e. the «classical» skin epithelium and the "non-classical" epithelium of pancreatic islets (Fig. 5).

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References


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