A Critical Role for ras-mediated, Epidermal Growth Factor Receptor-dependent Angiogenesis in Mouse Skin Carcinogenesis

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INTRODUCTION

Mouse and human keratinocytes are critically dependent on EGFR signaling (1). In vivo studies have demonstrated that a lack or impairment of EGFR function in skin leads to important mesenchyma-epithelium alterations. One of the most striking of these is the disruption of normal hair development (2–4). Both human and mouse epithelial tumors show EGFR overexpression (5, 6), and the abrogation of EGFR function inhibits the development of mouse skin tumors. However, it is unclear which signaling pathways downstream of the EGFR specifically control such inhibition. In fact, a reduction in the size of papillomas derived from EGFR-null keratinocytes expressing v-rasH4 in grafting experiments has been reported (7). In addition, it has been shown that transgenic mice expressing an activated form of human Son of Sevenless (Sos-F) in the basal compartment of the epidermis and the outer root sheath of hair follicles (K5-SOS-F mice) develop spontaneous papillomas. When these animals are crossed with EGFR-null mice, the tumorigenic response is attenuated (8). Inhibition of skin tumor development is also obtained when K5-SOS-F mice are crossed with the naturally occurring mouse mutant strain waved-2, which is homozygous for a hypomorphic EGFR allele (4). Increased apoptosis was suggested as the underlying cause of such inhibition. These studies, however, did not take into account the changes taking place at the stromal level, including angiogenesis, which is known to be critical in tumor growth (9, 10).

We have shown that the development of papillomas is preceded by a burst of angiogenesis and that an immature blood vessel network is established as tumors progress (11). In this respect, activation of Ha-ras appears to be not only the critical event in tumor initiation but also a main player in the mouse tumor angiogenic response (12), in which VEGF has a major role (13). We and others have shown that Ha-ras activation induces VEGF expression in mouse keratinocytes (12) and other cell types (14, 15). The importance of VEGF up-regulation in mouse skin carcinogenesis has also found support in studies from our group that show accelerated tumor development in transgenic mice expressing VEGF under the control of an epidermal promoter (16).

It has been reported that EGF induced the expression of VEGF in human keratinocytes in vitro (17) and also that the use of an anti-EGFR neutralizing antibody in A431 human epidermoid cells induces a dose-dependent inhibition of VEGF expression (18).

The present study investigates whether EGFR signaling regulates the changes observed in the skin tumor stroma, e.g., angiogenesis. This study reports that ras activation induces EGFR up-regulation, which in turn appears to modulate the angiogenic factor expression profile. Furthermore, we show that tumor growth inhibition resulting from functional EGFR abrogation in the presence of activated ras is associated with angiogenesis inhibition that can be rescued by forced expression of VEGF. We thus provide evidence that EGFR plays a central role not only in the regulation of key epidermal cell molecules but also in the establishment of stromal changes leading to skin tumor growth.

MATERIALS AND METHODS

Transgenic Mice. The animals used were the K5-HERCD-533 mice described previously (2). In this work, they are referred to as K5.dnEGFR mice. Constructs. The pK5-HERCD-533 construct has been described previously (2). Plasmid pcDNA3.dnEGFR was constructed as an Asp-718/HindIII fragment containing the dn EGFR cDNA (HERCD-533; Ref. 19) cloned into pcDNA3 vector (Invitrogen, Groningen, the Netherlands). Mouse VEGF164 cDNA was excised as an EcoRI fragment from plasmid pVEGF1 (20) and cloned in pCEP4 vector (Invitrogen). To construct the pK5.hEGFR plasmid, wt hEGFR cDNA was excised as a SacII/XhoI fragment from plasmid pCNV-3402.
EGFR-DEPENDENT ANGIOGENESIS AND SKIN TUMOR GROWTH

RESULTS

Activated Ha-ras Induces the Expression of EGFR mRNA in Mouse Epidermal Tumor Cells. In vivo studies have demonstrated increased expression of EGFR in mouse epidermal tumors (6). To investigate the relationship between EGFR expression and malignant behavior, Northern blots were performed to examine EGFR expression in a set of mouse epidermal cell lines representing different stages of mouse skin carcinogenesis (12). Tumorigenic cell lines with higher levels of activated ras expression and more tumorigenic also showed higher levels of EGFR expression (HaCa4 > PDVC57 > PDV). In nontumorigenic PB keratinocytes, which do not contain an activated ras, the expression of neither EGFR mRNA (Fig. 1A) nor protein (data not shown) was detected.

Remarkably, the forced expression of v-ras by retroviral transduction of PB cells induced a strong overexpression of EGFR mRNA (Fig. 1A). To our knowledge, this is the first time the induction of EGFR by Ha-ras has been described, and, as discussed later, this plays a critical role in ras-driven mouse skin carcinogenesis.

The Expression of Angiogenic Factors Depends on EGFR Activity. Previous work from our group demonstrated that VEGF expression, both in vivo and in vitro, correlates with Ha-ras activation level in mouse skin tumors (12). To study whether the Ha-ras-dependent angiogenic response was mediated through the above-described EGFR up-regulation, the function of the EGFR in EGFR-deficient PB cells was restored by permanent transfection with the pK5.hEGFR expression vector. Upon incubation with EGF, transfected PB cells expressing exogenous EGFR (PBhEGFR cells) induced the expression of VEGF mRNA (Fig. 1B).

In addition to VEGF as a proangiogenic factor in tumors, other positive or negative regulators released by both tumor and host cells mediate tumor angiogenesis (24, 25). It has been reported that Ang-1 may act as a stabilizing, angiostatic factor (26), and we have found that mouse skin tumors and epidermal tumor cells expressing activated Ha-ras abrogate the expression of Ang-1. To determine whether, as for VEGF expression induction, the inhibition of Ang-1 expression in activated-Ha-ras expressing cells was also EGFR dependent, two complementary experiments were performed. First, highly tumorigenic v-Ha-ras-carrying HaCa4 cells expressing high levels of EGFR were transfected with a plasmid carrying a dn EGFR cDNA shown to abrogate EGF function in vitro and in vivo (2). Strong induction of Ang-1 was observed in the dn EGFR-transfected cells compared with HaCa4 cells transfected with an empty plasmid (Fig. 1C). Lack of Ang-1 mRNA expression in HaCa4 cells that contain remarkably elevated levels of activated viral ras and EGFR mRNAs is consistent with their tumorigenic properties. Although forced expression of dn EGFR in HaCa4 cells strongly induced Ang-1 expression, the levels of v-ras remained unaltered (Fig. 1C). This result points to the upstream EGFR as the major Ang-1 regulator, independent of constitutive ras activation.

In the second experiment, forced expression of activated ras in-
duced EGFR expression in the nontumorigenic PB cells (which do not express EGFR but do constitutively express Ang-1) and abrogated Ang-1 mRNA (Fig. 1, A and C). This is in agreement with the acquisition of tumorigenic capacity by PB cells transfected with wt hEGFR (Fig. 1D; see below).

Thus, these results suggest that in addition to its critical role in epidermal cell physiology, EGFR acts as a major regulator of the stromal/vascular response in epidermal tumors. To further assess the importance of EGFR in the tumor angiogenic response, the in vitro results were validated in ex vivo skin carcinogenesis experiments.

**EGFR Functional Status Determines Cell Transformation and s.c. Tumor Growth.** To provide functional evidence of the role of EGFR in the acquisition of the malignant phenotype, the effect of forced expression of this receptor on the tumorigenic behavior of PB cells was analyzed. As mentioned above, this cell line is not tumorigenic when injected s.c. into nude mice. PB cells were cotransfected with a plasmid containing wt hEGFR cDNA (pK5.hEGFR) together with a neo-containing plasmid (pcDNA3), and the neo-resistant pooled clones (PBhEGFR) were injected into nude mice. PB cells transfected with the pcDNA3 vector were used as a control. Five weeks after injection, control PB cells gave rise only to small, whitish cysts (~200 mm³), as reported previously for wt PB cells (Ref. 21; Fig. 1D, top panel). Histological analysis indicated that these lesions were poorly vascularized, differentiated epidermoid cysts. In contrast, PBhEGFR cells developed into large tumor masses (~2.5–3.0 cm³) with a distinctive red appearance suggestive of robust vascularization (Fig. 1D, bottom panel). Histologically, these tumors were diagnosed as highly vascularized, moderately differentiated SCCs.

To confirm and extend these findings, experiments were performed to see whether the abrogation of EGFR function in a tumor epidermal cell line results in a loss of tumorigenicity. The highly tumorigenic PDVC57 cell line was therefore transfected with the dn EGFR construct. The mutant Ha-ras-carrying PDVC57 cell line was chosen because it expresses high levels of EGFR and produces differentiated carcinomas in the nude mouse assay reminiscent of those produced by the two-stage chemical skin carcinogenesis in vivo (27). Three different pools of neo-resistant dn EGFR-containing clones, designated PDVC57dn1, PDVC57dn4, and PDVC57dn5, were expanded and characterized. The expression of dn EGFR was confirmed by immunofluorescence (data(639,605),(870,840) not shown), and inhibition of the EGFR function was verified by the loss or decrease of EGFR autophosphorylation after EGF stimulation of the different PDVC57dn clones (data not shown). Because the different pools of clones showed similar properties, they were referred to generically as PDVC57dn cells, whereas those containing the empty plasmid were named PDVC57wt. Groups of nude mice injected with either PDVC57wt or PDVC57dn cells developed tumors of similar size when examined 1 week after cell injection. However, from this point, tumors that originated from PDVC57wt cells showed faster and more sustained growth than those that originated from PDVC57dn cells (Fig. 2, A and B). By the time the animals were sacrificed (3 weeks after injection), the PDVC57dn-derived tumors were notably smaller than the PDVC57wt-derived tumors (Fig. 2, A and B). Analysis of phosphorylation indicated the absence of EGFR function in the PDVC57dn tumors (data not shown). In addition to size differences, the reddish appearance was a remarkable feature of tumors derived only from PDVC57wt cells. Histological analysis revealed that tumors from both groups were moderately differentiated to well-differentiated SCCs, although wt tumors showed areas of increased differentiation.

**Impaired Angiogenic Response in Tumors Derived from dn EGFR-expressing Cells.** The pale appearance of the dn EGFR tumors (from PDVC57dn cells) pointed to a deficient blood supply as a possible cause of growth inhibition. Analysis of the vasculature in tumors at day 21 after injection using CD31 staining showed no major differences in vascular density between wt and dn EGFR tumors. However, important differences were observed when vessel morphology was analyzed (Fig. 3, A and B). dn carcinomas showed a pattern of blood vessels characterized predominantly by small and narrow capillaries (Fig. 3B). In contrast, wt tumors showed a network of dilated immature vessels (Fig. 3A). To establish the kinetics of vessel changes, the angiogenic response was studied at different times of carcinoma development (days 8, 11, and 14 after cell injection). Analysis of CD31 staining showed that, until day 8 after injection, when wt and dn tumors grow at the same rate, both kinds of carcinomas had blood vessels of similar size and appearance. At days 11 and 14 after injection, a progressive increase in the number of lacunar vessels appeared in the wt tumors concomitant with the increase in tumor growth rate (Fig. 3, C–E).

To investigate the molecular changes involved in the modulation of angiogenesis by the EGFR, the angiogenic factor profile expression of dn and wt tumors was examined at the mRNA level. In fully developed tumors (day 21 after injection), a marked difference in VEGF expression was observed between wt and dn EGFR tumors (Fig. 3F). The low VEGF mRNA levels observed in dn EGFR tumors were similar to those found in normal mouse skin.

Northern blot analysis of mRNA extracted at different times during tumor development (Fig. 3G) showed that VEGF mRNA levels in wt tumors increased progressively from day 8 onward in parallel with the increase in blood vessel diameter (Fig. 3, C–E).

**Fig. 2.** Ex vivo tumor development of wt and dn EGFR-expressing transformed mouse keratinocytes. A, physical appearance of tumors 21 days after injection of PDVC57wt (right flank) and PDVC57dnEGFR (left flank) cells into nude mouse. B, kinetics of tumor growth after cell injection.
expression of VEGF was forced in the PDVC57dn cells by transfection with a plasmid containing VEGF164 cDNA (Fig. 5). Isolated clones were named dnVEGF. Western blot analysis showed that VEGF did not alter the state of EGFR phosphorylation (data not shown). The majority (~75%) of the dnVEGF tumors had a size and physical appearance similar to those of wt carcinomas (Fig. 2A and Fig. 5, A and B). CD31 staining of these tumors demonstrated a pattern of blood vessels with enlarged lumen similar to those found in wt tumors (data not shown).

In Vivo Growth of Papillomas Is Also Associated with Altered Tumor Angiogenesis. Skin carcinogenesis experiments were performed using K5.dnEGFR transgenic mice, which express the dn EGFR mutant in the basal layer of the epidermis and show striking alterations in hair follicle development and hair cycling regulation (2). Tumors were induced by TPA treatment in F1 crosses of K5.dnEGFR mice with a transgenic strain (Tg.AC) carrying a mutant Ha-ras transgene that acts as the classic initiation event (16). Experiments were repeated three times using two different transgenic lines representative of different dn EGFR transgene integration sites and copy numbers (lines T4 and T0). Line T0 mice exhibited the more severe skin phenotype (2). Macroscopic analysis of tumors at all times during and after TPA treatment revealed that tumor incidence (the percentage of animals with tumors) and tumor multiplicity (number of tumors/mouse) were slightly reduced in both transgenic mouse lines (Table 1). By the time tumors were first visible after 5–6 weeks of promotion, papillomas from both control and transgenic mice were of a similar size (data not shown). However, after 16 weeks of promotion, tumors from control mice had a much larger, reddish appearance, whereas those from K5.dnEGFR mice remained small and pale (Fig. 6A). At 30 weeks, when mice were sacrificed, papillomas of the dn EGFR group (T4 and T0 lines) showed no increase in size, remaining strikingly smaller than those from control littermates. TUNEL and anti-Ki67 immunostaining assays performed on papillomas sections showed a significant increase in apoptosis and a small decrease in proliferation that was not significant in dn tumors (Table 2). These in vivo results fully agree with those obtained in ex vivo experiments (Fig. 4A).

CD31 analysis of tumor vasculature demonstrated that, as with ex vivo-induced carcinomas from dn EGFR-transfected cells, papillomas developing in dn EGFR mice display a pattern of narrow vessels...
VEGF increases as well as other undetermined factors, a problem Subsequent vascular phenotypic changes may also rely on further loma progression is the enlargement of angiogenic blood vessels. whereas changes in vessel density occur at the early stages of papil- and dn EGFR tumors. In this regard, we and others have shown that mice. unaffected, further growth and progression become inhibited in these growth hypothesis. Thus, whereas early papilloma development is ments in K5.dnEGFR transgenic mice support the biphasic tumor vessel remodeling. The results from in vivo carcinogenesis approaches, abrogation of EGFR function was shown to result in a dramatic decrease in VEGF expression and an altered angiogen-esis response unable to properly nourish and oxygenate tumor cells. Increased apoptosis therefore appears to be the likely consequence of improperly vascularized tumor tissue.

DISCUSSION

In the present study, we demonstrate that disruption of the EGFR signaling pathway(s) impairs the in vivo growth of papillomas and carcinomas beyond a critical size, even in the presence of an activated Ha-ras. Moreover, we have established that a critical element in the impairment of tumor growth in the absence of functional EGFR is an impeded angiogenic response. In vitro experiments using epidermal cell lines allowed the establishment of a fundamental role of the EGFR as a modulator of the expression of VEGF and Ang-1.

Kinetic analysis of ex vivo tumor development indicated that carcino-va growth, arising from s.c. injection of transformed keratinocytes, is a biphasic process. The first phase, slow growth, occurs with low VEGF levels and is independent of EGFR function. In fact, a similar growth rate was seen during the first 8 days in both wt- and dnPVDC57-derived tumors, suggesting that the pre-existing blood vessels suffice to nourish tumors until they reach a critical size. Beyond this point, angiogenic responses mediated through EGFR appear to be an essential requirement for complete tumor growth. Other receptors of the EGFR family, such as HER2, also known to play relevant roles in carcinogenesis could be involved in this process because they can form heterodimers with the dn EGFR form used (see Ref. 2). This may account for a second phase of rapid growth requiring an angiogenic switch involving high VEGF levels and blood vessel remodeling. The results from in vivo carcinogenesis experi-mnts in K5.dnEGFR transgenic mice support the biphasic tumor growth hypothesis. Thus, whereas early papilloma development is unaffected, further growth and progression become inhibited in these mice.

A major finding was the striking reduction of VEGF expression and dramatic qualitative differences between the blood vessels of control and dn EGFR tumors. In this regard, we and others have shown that whereas changes in vessel density occur at the early stages of papil-loma formation (11, 28), the main vasculature change during papil-loma progression is the enlargement of angiogenic blood vessels. Subsequent vascular phenotypic changes may also rely on further VEGF increases as well as other undetermined factors, a problem currently under investigation at our laboratory.

The impairment of nutrient and oxygen supply in EGFR signal-impaired tumors may thus account for the decline in cell survival as measured by the increased apoptotic rate (Refs. 7 and 8; this study) with concomitant tumor growth inhibition. Support for this hypothesis is provided by the fact that the forced re-expression of VEGF in a dn EGFR-expressing tumor cell line restores normal tumor growth independently of Ang 1 levels after s.c. injection in immunodeficient mice. We attribute this effect to the high levels of VEGF expression in these tumors from the early stages of tumor formation (Fig. 7).

The present results indicate that a number of properties currently attributed to Ha-ras activation in mouse skin carcinogenesis can now be attributed to the EGFR signaling pathway. Although ras-dependent EGFR ligand up-regulation may contribute to cellular transformation through a putative autocrine loop (29, 30), the requirement for intact EGFR suggests that an additional signaling pathway other than the canonical ras/mitogen-activated protein kinase pathway is a necessary component of the ras transformation of epidermal cells. It has recently been proposed that EGFR-dependent pathways acting via phosphati-dylinositol 3'-kinase/Akt are key elements in mouse skin tumorigen-esis (8, 22). In fact, recent studies showed that the complex VEGF expression regulation also includes the phosphatidylinositol 3'-kinase/
Akt pathway (31, 32). In agreement with this, we found that carcino-
mas derived from PDVC57dn cells have a much lower Akt activity than those derived from wt cells (Fig. 4B).

dn EGFR impair not only the growth of benign papillomas in vivo but also the growth of SCCs, a clinically relevant result for the treatment of human epidermal neoplasia. Cancer therapies that incor-
porate EGFR targeting strategies combining EGFR blockade with radiation show increasing promise in preliminary reports of clinical trial (33). Therefore, elucidation of the critical downstream EGFR/ EGFR family-triggered events, which include angiogenesis, would greatly improve prevention and treatment efforts based on targeting these growth factor-receptor-signaling pathways.

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