Ras-induced interleukin-8 expression plays a critical role in tumor growth and angiogenesis

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Summary

The role of Ras oncogenes in promoting cellular transformation is well established. However, the contribution of Ras signaling to interactions between tumor cells and their host environment remains poorly characterized. Here, we demonstrate that the inflammatory mediator interleukin-8 (CXCL-8/IL-8) is a transcriptional target of Ras signaling. Using a tumor xenograft model, we show that Ras-dependent CXCL-8 secretion is required for the initiation of tumor-associated inflammation and neovascularization. Collectively, our data identify a novel mechanism by which the Ras oncogene can elicit a stromal response that fosters cancer progression.

Introduction

Tumor development is driven by the sequential acquisition of genetic alterations involving the constitutive activation of protooncogenes and the loss of function of tumor suppressor genes. These changes confer a specific set of cellular traits that release cells from their normal growth constraints. Although many tumor characteristics can be attributed to the genetically altered cell itself, it is becoming increasingly clear that the ability of malignant cells to interact with and influence their tissue environment is critical for cancer development. For example, during tumor progression, cancer cells activate stromal fibroblasts and recruit inflammatory cells, which remodel the tumor stroma in part through the secretion of proteases (Coussens et al., 1999; Lin et al., 2001; Liotta and Kohn, 2001). This, in turn, leads to the release of growth-promoting factors sequestered in the extracellular matrix (ECM) and liberates neoplastic cells from the constraints of contact inhibition (MacDougall and Matrisian, 1995; Egeblad and Werb, 2002). In addition, tumor-associated inflammation also appears to play a key role in the initiation of angiogenesis as suggested by the findings that infiltration of mast cells at the tumor site activates premalignant neovascularization (Coussens et al., 1999).

About 25% of all human neoplasms contain mutationally activated forms of the Ras protooncogene (Bos, 1989). Ras proteins exert their tumorigenic effects through the activation of an intricate signaling network consisting of multiple downstream effectors (Campbell et al., 1998). A central role in transmitting proliferative signals is performed by the Ras effector Raf-kinase, which activates the ERK-MAP-kinase cascade (Downward, 1997). ERK, in turn, phosphorylates and activates transcription factors, thereby inducing cell cycle regulatory genes required for entry into S phase of the cell cycle (Marais et al., 1993; Gille et al., 1995). The initiation of survival signals, on the other hand, is primarily mediated by the interaction of Ras with the catalytic subunit of phosphoinositol 3-kinase (PI3K) (Kauffmann-Zeh et al., 1997). This interaction leads to the activation of the serine/threonine kinase Akt/PKB, which in turn augments cell survival by suppressing or enhancing pro- or antiapoptotic functions, respectively (Brazil et al., 2004). In addition, Ras signaling contributes to the metastatic properties of tumor cells through the activation of effector pathways involving Rho GTPases. Activation of these GTPases promotes cell motility by inducing specific changes in the organization of the actin cytoskeleton (Schmitz et al., 2000).

Apart from these cell-autonomous effects, Ras oncogene expression promotes and sustains tumor-host interactions that are essential for neoplastic development. Constitutive Ras activity has been shown to contribute to increased tumor cell invasiveness through the activation of matrix metalloproteinases, which initiate basement membrane degradation (Ballin et al., 1988). Furthermore, Ras is known to promote endothelial cell-dependent tumor angiogenesis, mainly by means of transcriptional upregulation of vascular endothelial growth factor (VEGF) and through the repression of the antiangiogenic protein thrombospondin-1 (TSP-1) (Okada et al., 1998; Rak et al., 2000; Wat-

SIGNIFICANCE

The instructive interactions between tumor cells and their microenvironment are now recognized as essential determinants in the development of malignancy. In particular, the recruitment of blood vessels to the tumor site is a prerequisite of tumor growth and metastasis. In the present study, we show that the transcriptional upregulation of the chemokine interleukin-8 (CXCL-8/IL-8) by oncogenic Ras is required for the onset of tumor vascularization. Our findings underscore the contribution of dysregulated CXCL-8 expression to the process of Ras-mediated tumorigenesis and suggest that this chemokine and its receptors may serve as attractive anticancer therapeutic targets.

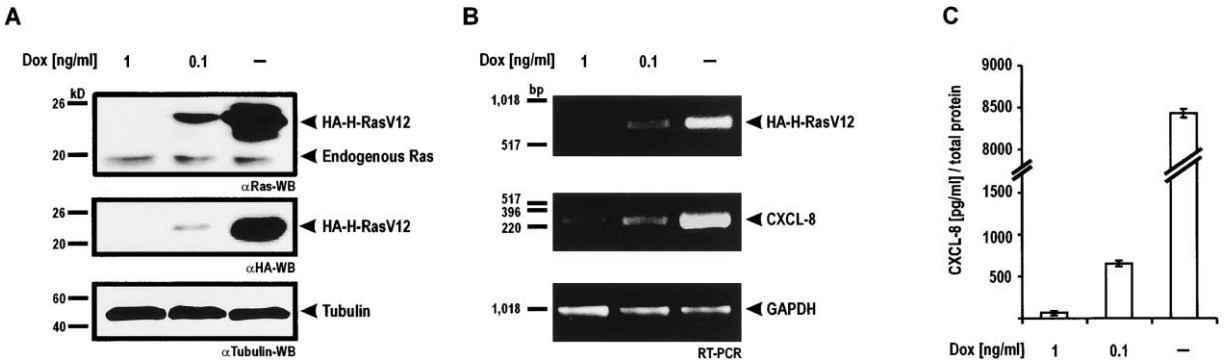

Figure 1. Expression of H-RasV12 induces CXCL-8 transcription and protein synthesis

HeLa Tet-Off H-RasV12 transgenic cells (HTO H-RasV12) were cultured with the indicated concentrations of doxycycline 24 hr prior to lysis.
A: Total cell extracts were used to detect both exogenous and endogenous Ras protein by Western blot analysis.
B: RNA was isolated and subjected to RT-PCR analysis using specific primers to the indicated genes.
C: Tissue culture supernatants were analyzed by human CXCL-8-specific ELISA. Results are reported as the mean ± SD of three independent experiments each run in duplicate.

nick et al., 2003). However, the signaling mechanisms that mediate the effects of Ras on the interactions between tumor cells and their host environment remain poorly understood.

In the current study, we have analyzed the functional significance of Ras-mediated induction of the proinflammatory chemokine interleukin-8 (CXCL-8/IL-8). Using a nude mouse xenograft model, we demonstrate that CXCL-8 synthesis and secretion is required for RasV12-stimulated tumor inflammation, vascularization, and growth. These observations suggest a novel signaling mechanism by which neoplastic cells can trigger angiogenesis through the recruitment of immune cells to tumor sites.

Results

Induction of interleukin-8 by RasV12

To identify Ras target genes, we performed high-density oligonucleotide-based microarray analyses in HeLa cell lines expressing activated H-RasG12→V transgenes under a tetracycline responsive promoter (Tet-Off Expression System). Among all targets represented on the Affymetrix GeneChip, mRNA transcripts of interleukin-8 (CXCL-8/IL-8), a well-characterized proinflammatory chemokine, exhibited the most substantial (~63-fold) upregulation in response to H-RasV12 expression. This observation is in agreement with a recent study, in which RasV12 expression in human ovarian cells was shown to induce various cytokines involved in inflammatory responses, including CXCL-8 (Liu et al., 2004).

To validate the results from the global gene expression analysis, we examined the induction of CXCL-8 by RT-PCR and ELISA. A significant increase in CXCL-8 mRNA and protein levels (Figures 1B and 1C) was observed in response to both near-physiological and supraphysiological levels of H-RasV12 (Figure 1A). To exclude cell type-specific effects of Ras on CXCL-8 expression, we have analyzed the induction of CXCL-8 by H-RasV12 signaling in two additional human cell lines of epithelial origin using adenoviral gene delivery. As illustrated in Figure 2, H-RasV12 increased CXCL-8 expression in both a lung carcinoma cell line (H125) and a normal, spontaneously immortalized human lumenal mammary epithelial cell line (MCF-10A). The mammalian genome encodes four Ras isoforms, which, despite their high homology, appear to possess unique signaling properties and different oncogenic potential (Bos, 1989; Bar-Sagi, 2001). To determine whether the ability to induce CXCL-8 expression reflects a unique property of H-Ras, we infected cells with an adenoviral construct that directs the expression of constitutively active K-Ras. Similar to H-Ras, signaling from the K-Ras isoform led to an increase in both CXCL-8 mRNA and protein (Figure 2). It is noteworthy that the levels of CXCL-8 expression varied between the different cell types. Presumably, these differences are dictated by cellular context, as the infection efficiencies were similar for each of the various cell lines (Supplemental Figure S1 at http://www.cancercell.org/cgi/content/full/6/4/447/DC1/). Overall, these results indicate that the signaling pathways leading from Ras to CXCL-8 upregulation are functional in both tumor-derived and normal human cells and conserved between at least two Ras isoforms.

Ras-mediated transcriptional upregulation of CXCL-8 requires the concurrent activation of both MAPK and PI3K effector pathways

The induction of CXCL-8 message coincided with H-RasV12 protein expression, suggesting an immediate effect of Ras-mediated signaling on CXCL-8 transcription (Supplemental Figure S2 at http://www.cancercell.org/cgi/content/full/6/4/447/DC1/). To analyze the role of different Ras effector pathways in CXCL-8 transcription, we utilized a reporter construct in which the minimal CXCL-8 promoter region was fused to the firefly luciferase gene (Wu et al., 1997). Constitutive activation of the ERK cascade through a dominant active mutant of Raf, Raf-CAAX, or of the PI3K pathway through the membrane-targeted catalytic subunit of PI3K, p110CAAX, led to partial activation of the CXCL-8 promoter (Figure 3A). However, simultaneous activation of both pathways through the coexpression of Raf-CAAX and p110CAAX increased luciferase activity to the levels reached by H-RasV12 expression. This indicates that maximal

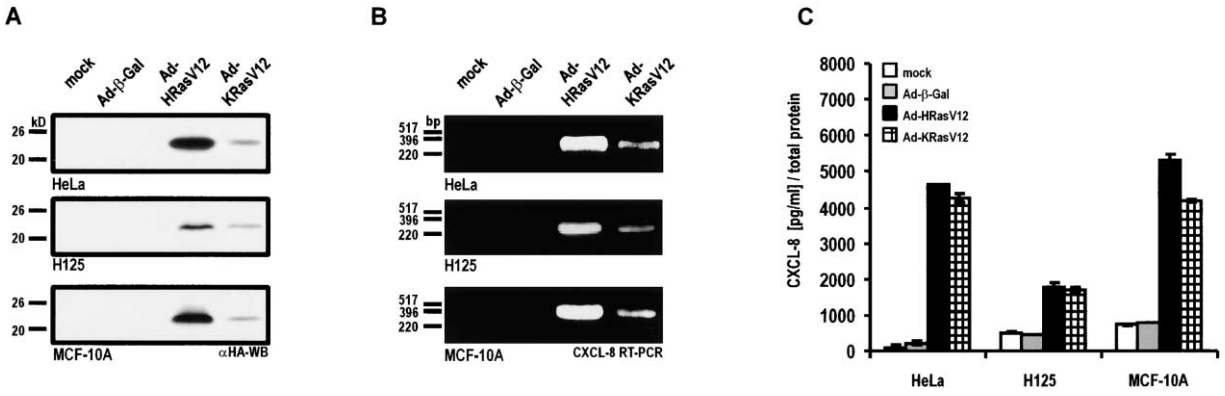

Figure 2. RasV12-induced CXCL-8 expression in different human cell lines

Cervical cancer cells (HeLa), lung carcinoma cells (H125), and normal breast epithelial cells (MCF10A) were infected with recombinant adenovirus that directs the expression of either β-galactosidase (Ad-β-Gal), HA-epitope-tagged H-RasV12 (Ad-HRasV12), or K-RasV12 (Ad-KRasV12) as indicated. All samples were harvested 24 hr after infection.

A: Cytoplasmic lysates were used to detect expression of the HA-RasV12 transgenes with an antibody raised against the HA-epitope tag.

B: RNA was isolated and subjected to RT-PCR using CXCL-8-specific primers.

C: Tissue culture supernatants were analyzed by human CXCL-8-specific ELISA analysis. Results are reported as the mean ± SD of three independent experiments, each run in duplicate.

induction of CXCL-8 gene expression depends on the concurrent activation of both Ras effector pathways. The contributions of these signaling cascades to Ras-mediated CXCL-8 induction were further confirmed by demonstrating that the pharmacological inhibitors U0126 and LY294002, which specifically target the ERK-MAPK and the PI3K pathway, respectively, abrogated CXCL-8 induction by H-RasV12 (Figure 3B).

We next sought to dissect the pathways downstream of PI3K involved in the induction of CXCL-8. A central role in PI3K-mediated signaling is performed by the serine/threonine kinase Akt/PKB (Franke et al., 1997). Additionally, there is growing evidence that the small GTPase Rac is regulated by PI3K-dependent mechanisms (Welch et al., 2003). To differentiate between the contributions of Akt or Rac to the activation of CXCL-8 transcription, we attempted to mimic PI3K signaling by expressing either a mutant of Akt that is rendered constitutively active through a myristoylation sequence at its carboxy-terminus (myrAkt), or a dominant active mutant of Rac, RacV12. Expression of either myrAkt or RacV12 led to a moderate increase in luciferase activity (Figure 3A). To ascertain that in our experimental system, Rac signals downstream of PI3K, we coexpressed a dominant interfering mutant of Rac, RacN17, together with p110CAAX. RacN17 partially inhibited PI3K-induced CXCL-8 promoter activity, indicating the involvement

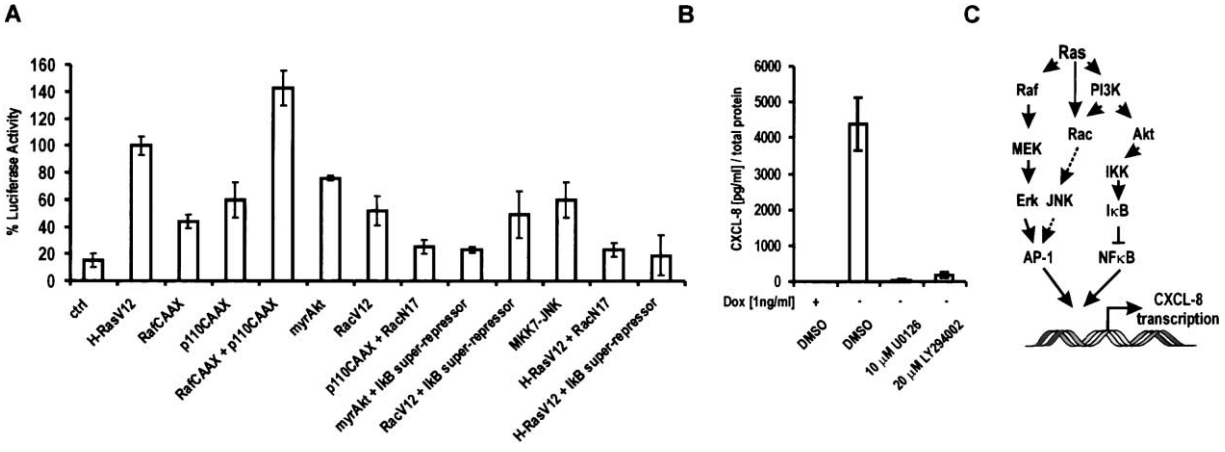

Figure 3. Analysis of transcriptional regulation of the CXCL-8 promoter by RasV12 signaling in HeLa cells

A: HeLa cells were cotransfected with a CXCL-8-luciferase reporter construct and the indicated expression plasmids. Luciferase assays were conducted 24 hr after transfection. Results are reported as the mean ± SD of three independent experiments performed in triplicate and normalized for transfection efficiency using β-galactosidase.

B: Tissue culture supernatants from HTO H-RasV12 cells cultured in the presence (+) or absence (−) of 1 ng/ml doxycycline for 16 hr were collected and analyzed using CXCL-8-specific ELISA. Selective inhibitors of MEK (U0126) and PI3K (LY 294002) were added at the indicated concentrations at the time of induction. Values represent the mean ± SD of three independent experiments performed in duplicate.

C: Schematic diagram of the signaling pathways mediating Ras-induced transcriptional upregulation of CXCL-8.

of Rac in the induction of CXCL-8 by PI3K (Figure 3A). Akt and Rac both stimulate the transcription factor nuclear factor κB (NFκB) (Perona et al., 1997; Romashkova and Makarov, 1999). Since NFκB is an established activator of the CXCL-8 promoter (Matsusaka et al., 1993), we aimed to determine whether the effects of Akt and Rac on CXCL-8 promoter activity are mediated through this transcription factor. To this end, we coexpressed myrAkt or RacV12 with a dominant interfering mutant of the NFκB cascade, the inhibitor of NFκB (IκB) super-repressor protein, which is a nondegradable form of IκB that binds to NFκB and prevents its translocation into the nucleus (Brockman et al., 1995). Only myrAkt-mediated induction of luciferase activity could be inhibited by the IκB super-repressor, indicating that Akt acts upstream of NFκB to induce CXCL-8 transcription, while RacV12 activates the CXCL-8 promoter through an alternative pathway (Figure 3A). One additional mechanism mediating the effects of Rac on transcription is the activation of the c-Jun N-terminal (JNK) kinase cascade (Coso et al., 1995). Expressing a construct in which JNK is rendered constitutively active through a fusion to the activation domain of MKK-7 (Lei et al., 2002) led to partial activation of the CXCL-8 promoter, suggesting that activation of JNK might play a role in the induction of CXCL-8 transcription (Figure 3A). That Rac and NFκB are necessary for Ras-induced CXCL-8 expression was further established by the finding that either RacN17 or the IκB super-repressor protein inhibited H-RasV12 induced activation of the CXCL-8 promoter (Figure 3A).

Neutralization of CXCL-8 in RasV12-expressing subcutaneous tumors attenuates neoplastic growth

We next sought to determine whether the induction and secretion of CXCL-8 in response to RasV12 signaling contributes to Ras-mediated tumorigenesis. To this end, we conducted xenograft studies using immunocompromised nude mice. Animals were divided into two experimental groups. The first cohort was injected subcutaneously with H-RasV12 transgenic HTO cells cultured in the presence of doxycycline to repress expression of H-RasV12. In vivo suppression of H-RasV12 transcription in these cells was maintained by supplementing the drinking water with 2 mg/ml doxycycline. The second cohort received HTO H-RasV12 cells induced to express H-RasV12 through removal of doxycyline 24 hr prior to introduction into the host. The regulation of H-RasV12 transgene expression in vivo was confirmed by immunoblot analysis of tumor lysates (data not shown). Animals were monitored for subcutaneous tumor formation and the growth rate of the developing tumors was established. As illustrated in Figure 4A, H-RasV12 expression significantly enhanced tumor growth in experimental animals. ELISA analysis of serum taken from experimental animals at different time points after tumor cell inoculation revealed the presence of CXCL-8 in the bloodstream of mice injected with H-RasV12-expressing cells at levels that appear to correlate with tumor size (Supplemental Figure S3 at http://www.cancercell.org/cgi/content/full/6/4/447/DC1/).

To investigate the contributions of CXCL-8 to H-RasV12-induced tumor growth, we inhibited CXCL-8 function using a CXCL-8 neutralizing antibody, MAB208 (R&D Systems). This approach has been applied successfully to document the role of CXCL-8 in proliferation and subcutaneous growth of Kaposi's sarcoma-derived tumor cells (Masood et al., 2001). Antibody injections at the tumor site were initiated two days after tumor cell inoculation, at which point palpable nodules had developed, and were thereafter continued twice a week. The efficiency of CXCL-8 targeting by this protocol is indicated by the absence of detectable CXCL-8 levels in the serum of animals injected with the CXCL-8 neutralizing antibody at every stage of tumor development (Supplemental Figure S3). Whereas no significant differences were observed between tumor size from isotype-matched (IgG_1) control antibody treated and untreated groups, animals injected with the CXCL-8 neutralizing antibody demonstrated an approximate 60% reduction in tumor growth rate at the time of sacrifice when compared with animals bearing control injected or noninjected tumors (Figures 4B and 4C). We confirmed that injection of either antibody did not interfere with H-RasV12 expression as demonstrated by transgene-specific immunoblot analysis of tumor lysates (Figure 4D).

To rule out the possibility that the effects of MAB208 are due to nonspecific in vivo crossreactivity, we employed a different CXCL-8 antibody, I8-6 (Antigenix America). Like MAB208, this antibody has potent CXCL-8 neutralizing activity as judged by a neutrophil chemotaxis assay (Supplemental Figure S4 at http://www.cancercell.org/cgi/content/full/6/4/447/DC1/). Significantly, introduction of I8-6 into H-RasV12 expressing tumors through the same injection regimen used for MAB208 inhibited Ras-induced tumor growth (Supplemental Figure S4). Together, these observations indicate that interference with CXCL-8 function curtails the tumorigenic potential of H-RasV12. In all subsequent analyses, tumors injected with IgG_1 antibody were indistinguishable from noninjected tumors and were therefore used as a control for tumors injected with the CXCL-8 neutralizing antibody.

CXCL-8 functions in a non-cell-autonomous manner

In principle, the effect of CXCL-8 could be mediated by autocrine and/or paracrine mechanisms. FACS analysis of the expression of the two CXCL-8 receptors CXCR-1 and -2 in H-RasV12-expressing HTO cells demonstrated that these cells did not express significant levels of either CXCL-8 receptor (Figure 5A). Moreover, neither receptor was expressed in the course of tumor development, as determined by RT-PCR analysis of human CXCR-1 and -2 in neoplastic tissue isolated at different time points after tumor cell inoculation (Figure 5B). These results argue against CXCL-8 functioning in an autocrine fashion in our experimental system. This interpretation is further supported by the observation that CXCL-8 did not exhibit a growth-stimulatory effect on HTO cells maintained either in growth factor-poor medium (0.5% serum) or in serum-containing medium (5% serum) (Figure 5C). Additionally, the migratory behavior of HTO cells was not influenced by the addition of CXCL-8, as measured by a modified Boyden chamber assay (Supplemental Figure S5). Collectively, these findings suggest that the role of CXCL-8 in tumor growth is mediated through nonautonomous effects on stromal cells.

Inhibition of CXCL-8 function does not affect tumor cell proliferation, but leads to an increase in tumor cell death and an impairment of tumor vascularization

To analyze the mechanisms underlying the requirement of CXCL-8 for Ras-induced tumor growth, we have examined whether CXCL-8 neutralization alters the proliferative status of H-RasV12-expressing tumor cells. End-stage tumors excised

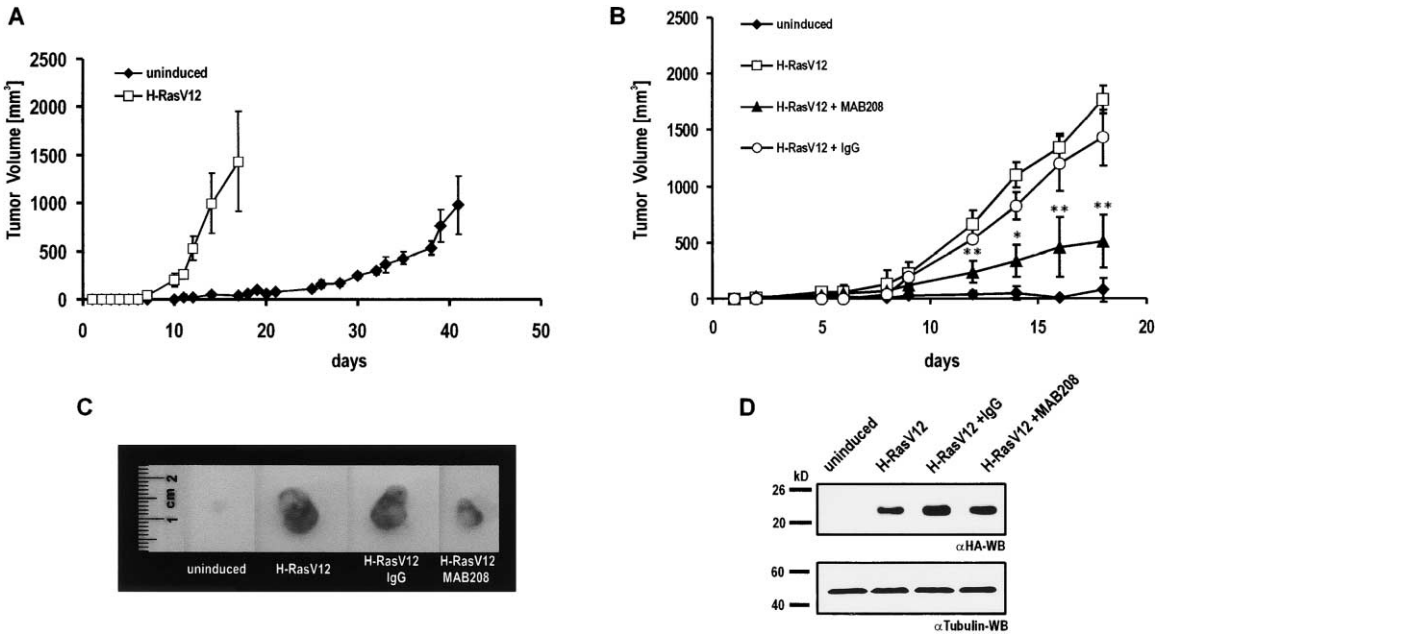

Figure 4. Inhibition of CXCL-8 activity in vivo attenuates RasV12-induced tumor growth

A: HTO H-RasV12 cells were cultured in the presence (uninduced) or absence (H-RasV12) of doxycycline for 24 hr and then subcutaneously injected into immunocompromised nude mice. The graphs indicate the mean tumor growth rates ± SD of three animals per experimental condition.

B: Nude mice were inoculated with HTO H-RasV12 cells, either repressed for transgene expression (uninduced) or expressing H-RasV12 (H-RasV12). Animals receiving H-RasV12-expressing HTO cells were randomized into three groups and either not injected or injected at the tumor site with 100 μg IgG_1 control antibody (IgG) or 100 μg neutralizing antibody to CXCL-8 (MAB208) on days 2, 6, 9, 13, and 16 after tumor cell inoculation. The graphs indicate the mean tumor growth rates ± SD of six animals per experimental condition (*$p < 0.05$; **$p < 0.01$).

C: Tumors were excised from animals at day 18 after tumor cell inoculation when the largest tumor had reached a diameter of 2 cm. Representative examples for each experimental group are shown.

D: Tumor tissue was lysed and HA-RasV12 protein was detected by immunoblot analysis using an antibody against the epitope-tag.

from the animals at day 18 postinoculation were immunostained for proliferating cell nuclear antigen (PCNA), a marker for cycling cells. H-RasV12-expressing tumors that were injected with the IgG_1 control antibody demonstrated an enhanced proliferative index (62 ± 8%) when compared to nonexpressing tumors (20 ± 11%). Injection with the CXCL-8 interfering antibody did not have a significant effect on the mitogenic rate of H-RasV12-expressing tumors (58 ± 10%) (Figure 6A). Thus, inhibition of tumor growth as a consequence of CXCL-8 inactivation cannot be attributed to a decrease in neoplastic cell proliferation.

In contrast, inhibition of CXCL-8 had a pronounced effect on tumor cell necrosis. Microscopic examination of H-RasV12-expressing tumors injected with CXCL-8 neutralizing antibody revealed areas of extensive necrosis, comprising 66% of total tumor area (Figure 6B). Necrosis was characterized by the presence of fragmented nuclear and cytoplasmic debris and a nearly complete lack of intact cells (Figure 6B, inset). In contrast, H-RasV12 expressing tumors injected with the isotype control antibody formed solid masses that had only sparse patches of less advanced necrosis encompassing approximately 30% of tumor area (Figure 6B).

We speculated that this substantial increase in tissue necrosis observed after ablation of CXCL-8 function was attributable to an impairment of angiogenesis, with tumor cell death occurring in response to hypoxic stress. To address this question, tumor tissue was evaluated histochemically using CD31 staining for tumor-associated vessels and quantitatively assessed for vessel number and diameter (Figure 6C). This analysis revealed a significant enhancement of vascularization in H-RasV12-expressing tumors injected with the IgG_1 control antibody as compared to the stunted vasculature found in uninduced tumors. Injection of H-RasV12-expressing tumors with MAB208 drastically reduced the increase in vascular density induced by H-RasV12 (Figure 6C). Since the two major determinants implicated thus far in Ras-induced angiogenesis, VEGF upregulation and thrombospondin-1 suppression (Okada et al., 1998; Rak et al., 2000; Watnick et al., 2003), are not affected by MAB208 injection (Figure 6D), we conclude that CXCL-8 function is specifically required for Ras-induced tumor vascularization.

CXCL-8-dependent recruitment of endothelial cells early in tumor development occurs coincident with stromal infiltration of inflammatory cells

To investigate whether CXCL-8 plays a role in the induction of angiogenesis during early stages of tumor development, we analyzed neoplastic tissue five days after cell inoculation, the earliest time point at which tumors reached a size that permitted their excision from the animals. Immunohistochemical staining with an antibody against the endothelial cell marker CD31 demonstrated the presence of CD-31-positive cells in H-RasV12-expressing tumors injected with the IgG_1 control antibody (Figure 7A). In contrast, endothelial cells were absent in MAB208 injected tumors, indicating that H-RasV12-induced CXCL-8 is

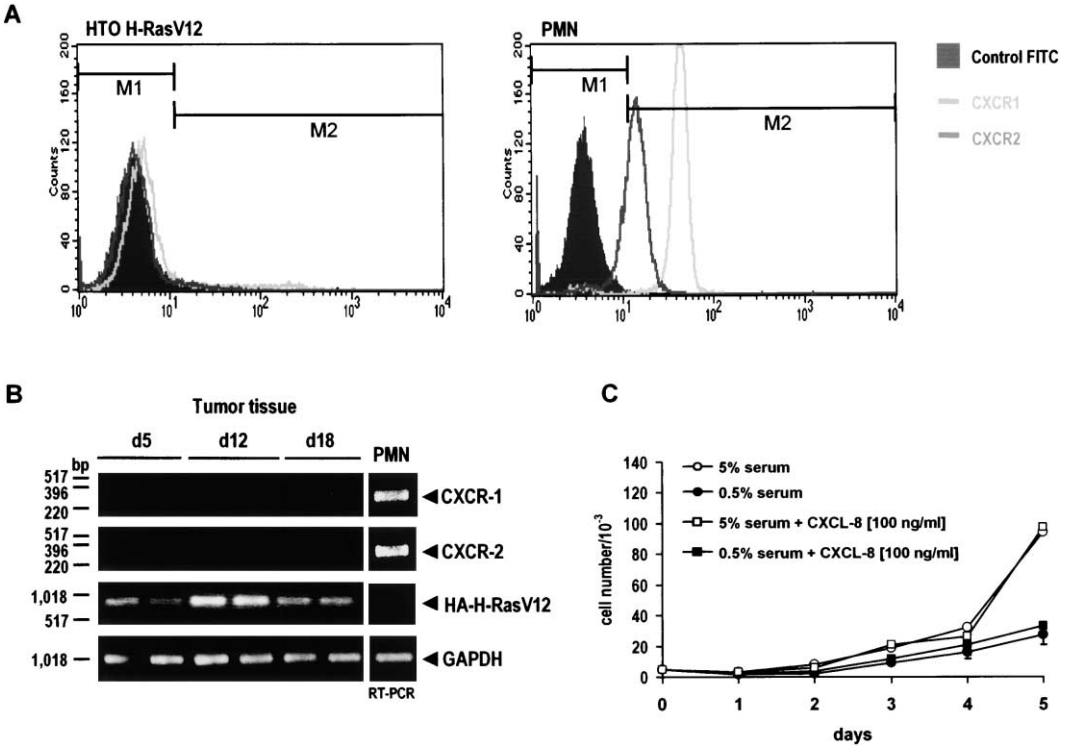

Figure 5. CXCL-8 does not function as an autocrine factor for HTO cells

A: H-RasV12-expressing HTO cells were assessed for CXCR-1 (light gray profile) or CXCR-2 (dark gray profile) expression by FACS analysis. Human polymorphonuclear neutrophils (PMN) served as positive control. Filled profiles show the labeling with isotype-matched IgG control antibody.

B: RNA was isolated from two independent tumors excised at day 5, 12, or 18 after tumor cell inoculation and subjected to RT-PCR analysis using specific primers to the indicated genes. Human PMN served as control.

C: HTO H-RasV12 cells were grown in the presence of 1 ng/ml doxycycline under the indicated conditions. Cell numbers were counted every 24 hr. Values shown represent the mean and standard deviation of three replicate experiments.

essential for the recruitment of endothelial cells to the tumor site (Figure 7A).

Because inflammation has been implicated in the initiation of tumor vascularization (Bergers et al., 2000), we investigated whether the recruitment of endothelial cells in H-RasV12-expressing tumors is linked to the presence of inflammatory cells. Immunohistochemical analysis using an antibody directed against α_M-integrin (MAC/CD11b) revealed infiltration of macrophages and granulocytes at the core of early-stage H-RasV12-expressing tumors injected with IgG_1 control antibody (Figure 7B). Notably, the recruitment of these types of cells has been shown to precede the activation of premalignant neovascularization (Coussens et al., 1999). Ablation of CXCL-8 function through injection of the CXCL-8 neutralizing antibody into H-RasV12-expressing tumors significantly reduced infiltration by inflammatory cells (Figure 7B), but had no effect on tumor cell proliferation (data not shown). These results demonstrate that early-stage H-RasV12-expressing tumors are dependent on CXCL-8 production for both the mobilization of endothelial cells and the development of an inflammatory response.

To further examine the cause-and-effect relationship between CXCL-8-induced inflammation and angiogenesis in H-RasV12-expressing tumors, experimental animals were subjected to i.p. injection of a monoclonal antibody directed against the myeloid differentiation antigen Gr-1/Ly-6-G (RB6-8C5, BD Biosciences). This antibody is cytotoxic for neutrophils and eosinophils and has been shown to lead to the effective depletion of these cell types in vivo (Sitia et al., 2002). As illustrated in Figures 7C and 7D, the injection of RB6-8C5 resulted in a significant attenuation of tumor inflammation and endothelial cell recruitment. Thus, the inflammatory response induced by CXCL-8 expression is required for the neovascularization of H-RasV12-expressing tumors.

Discussion

In the present study, we have characterized the functional significance of the upregulation of interleukin-8 (CXCL-8/IL-8) by oncogenic Ras. CXCL-8 induction is necessary for RasV12-induced tumor growth, and ablation of CXCL-8 function in RasV12-expressing tumors leads to a substantial decrease in tumor vasculature and extensive tissue necrosis. These findings indicate a role for CXCL-8 in Ras oncogene-dependent tumor angiogenesis.

CXCL-8 expression is induced by a wide variety of stimuli, including proinflammatory cytokines such as interleukin-1 or tumor necrosis factor, bacterial and viral products, and cellular stress. The transcriptional events necessary for the induction of CXCL-8 promoter activity have been extensively characterized (Hoffmann et al., 2002). In particular, the transcription factors AP-1 and NFκB have been shown to play a central role in the modulation of CXCL-8 expression (Yasumoto et al., 1992). Both

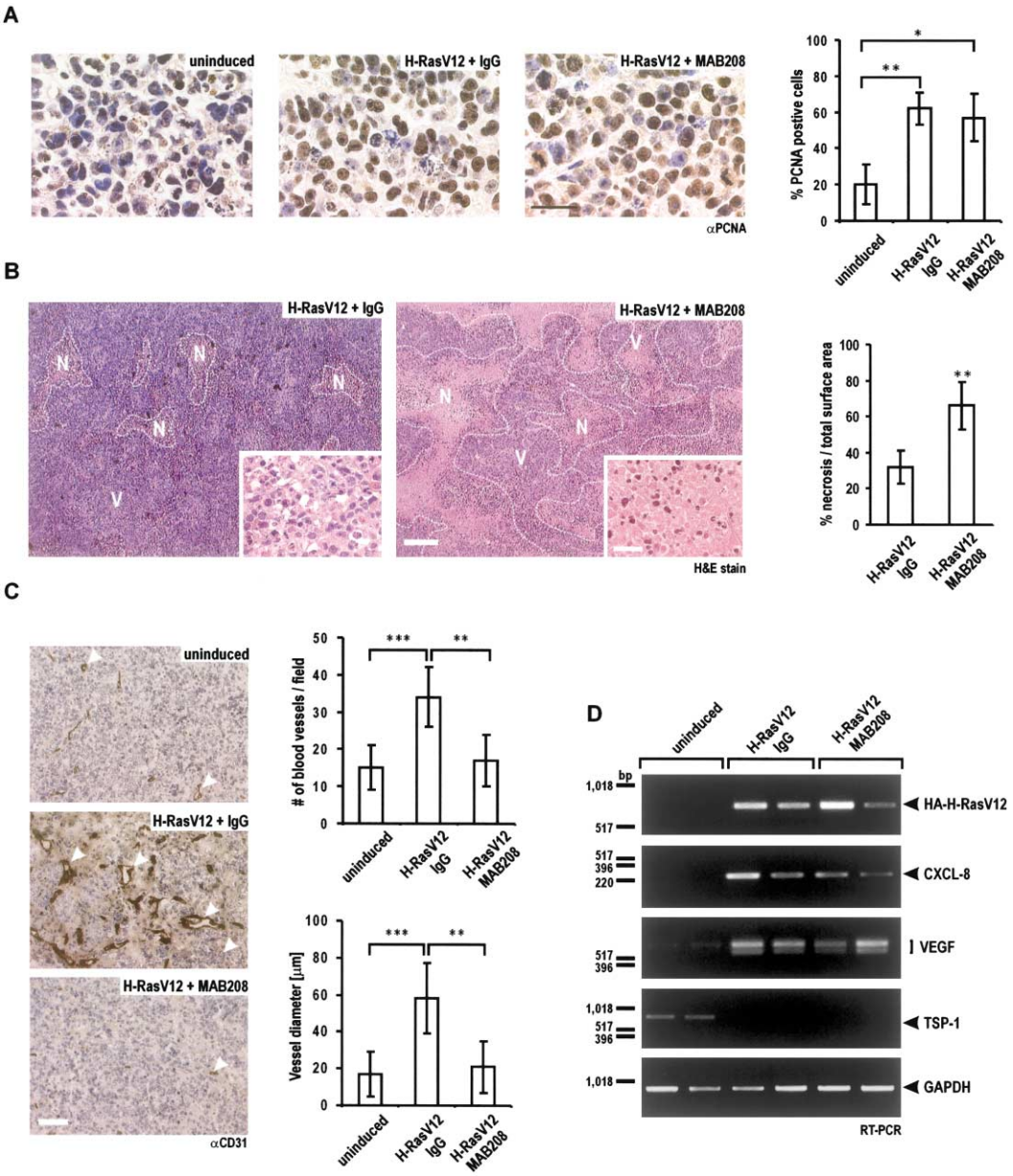

Figure 6. Inhibition of CXCL-8 function does not affect tumor cell proliferation, but leads to an increase in tumor cell death and an impairment of tumor vascularization

A: Uninduced or H-RasV12-expressing neoplastic tissues excised at day 18 from mice injected with control IgG_1 antibody or MAB208 as indicated were immunohistochemically stained using an antibody to PCNA. Representative examples of tumor sections stained with αPCNA are shown in the photomicrographs. Scale bar represents 20 μm. Results are shown as the percentages of PCNA positive cells in six high magnification fields per section. Each data point is derived from six tumors and corresponds to the mean ± SD (*$p < 0.05$; **$p < 0.01$).

B: Fixed sections of RasV12-expressing tumors excised at day 18 from mice injected with the IgG_1 control antibody or MAB208 were stained with H&E. Necrotic areas are marked with dashed lines. V denotes areas of viable tumor cells, N denotes areas of necrosis. Scale bar represents 200 μm. Insets are higher magnification (40×) images. Scale bar in insets corresponds to 20 μm. The surface area of necrotic tissue relative to the total surface area of the field of view was quantified by morphometric analysis. Values are the averages from six independent tumors per experimental condition. Standard deviations are indicated by error bars (**$p < 0.01$).

C: Sections of uninduced tumors, RasV12-expressing tumor injected with IgG_1 control antibody, or RasV12-expressing tumors injected with MAB208 were stained using an antibody against the endothelial cell surface marker CD31. Scale bar represents 50 μm. Blood vessel density and diameter was quantified by morphometric analysis. Values are the averages from six independent tumors per experimental condition. Standard deviations are indicated by error bars (**$p < 0.01$; ***$p < 0.001$).

D: RNA isolated from tumors excised at day 18 after tumor cell inoculation was subjected to RT-PCR analysis using primers to the indicated genes. The two distinct bands amplified by VEGF-specific primers represent splice variants of the VEGF-A gene. For each experimental condition, the two lanes represent two independent tumors.

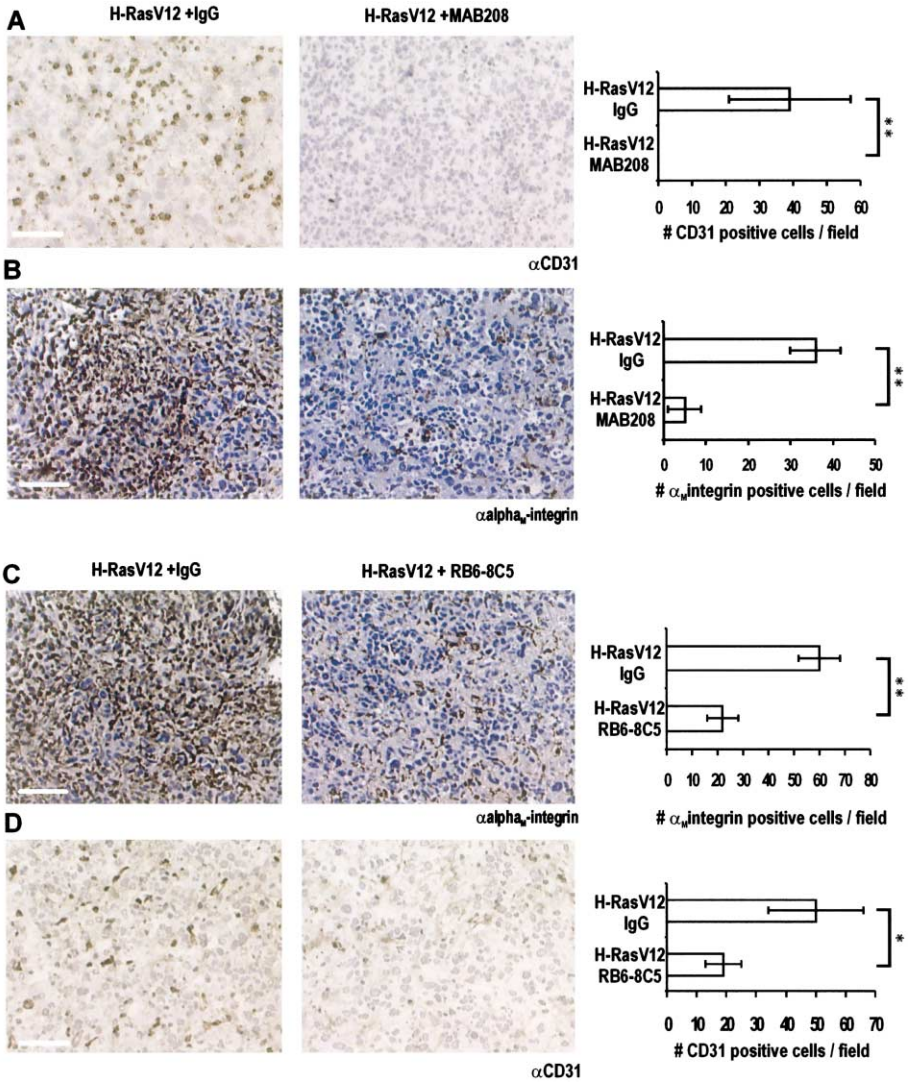

Figure 7. Endothelial cell recruitment during early stages of tumor growth correlates with infiltration of macrophages and granulocytes at the tumor site

Neoplastic tissue excised at day five after tumor cell inoculation from animals injected with IgG control antibody, MAB208 (**A** and **B**) or RB6-8C5 (**C** and **D**) was stained with an antibody directed against the endothelial cell marker CD31 or α_M-integrin (αMAC/CD11b). Photomicrographs show representative examples of three independent tumors analyzed per experimental condition. Scale bar represents 50 μm. Results are shown as the number of stained cells/field counting six high magnification fields per section. Each data point is derived from three independent tumors and corresponds to the mean ± SD (*$p < 0.05$;**$p < 0.01$).

of these transcriptional regulators are well-known targets of Ras signaling, and it has been shown that the integrity of the Ras pathway is required for ligand-mediated CXCL-8 expression (Zhao et al., 2001). We have established that oncogenic activation of Ras is sufficient to induce a pronounced increase in CXCL-8 expression. This effect is mediated by the coordinated activation of several Ras effector pathways, thus offering multiple potential targets for therapeutic intervention.

It is widely accepted that the growth of solid tumors beyond microscopic sizes is dependent on the development of an intact tumor vasculature. Among several growth factors that can function as positive regulators of angiogenesis, vascular endothelial growth factor (VEGF) is thought to play a central role by promoting the proliferation, survival, and migration of endothelial cells (Ferrara, 2002). It has been demonstrated that expression of oncogenic Ras results in the upregulation of VEGF (Arbiser et al., 1997; Rak et al., 1995). The signaling mechanisms underlying this response appear to be cell type-specific and involve the PI3K-pathway, the Raf/MAPK-cascade, hypoxia-inducible factor 1 (HIF-1) and/or several other possible mechanisms (Rak et al., 2000; Rak and Yu, 2004; Sodhi et al., 2001). In addition, Ras-dependent VEGF induction can be significantly influenced by physiological and environmental effects, such as high cell density and hypoxia (Mazure et al., 1996; Rak et al., 2000). VEGF has been shown to play an essential role in tumor angiogenesis meditated by Ras (Shi and Ferrara, 1999). However, under some circumstances, the upregulation of VEGF by Ras is clearly not sufficient for progressive tumor growth, as indicated by the inability of ectopically expressed VEGF to recapitulate the pro-angiogenic effects of oncogenic Ras (Chin et al., 1999; Okada et al., 1998). An additional mechanism by which Ras signaling might promote angiogenesis is through the repression of the antiangiogenic protein thrombospondin-1 (TSP-1) (Rak et al., 2000). This notion is supported by the recent demonstration that the downregulation of TSP-1 expression by RasV12 is a critical step in the acquisition of tumor angiogenicity (Watnick et al., 2003). Our present finding that Ras-mediated CXCL-8 upregulation is necessary for tumor angiogenesis points to an additional mechanism through which Ras activation can trigger neovascularization. Thus, overall, the angiogenic phenotype elicited by oncogenic Ras likely depends on multiple signaling

events that act in concert to alter the balance between pro- and antiangiogenic factors.

In recent years, there has been growing appreciation for the potential role of tumor inflammation in the initiation of angiogenesis. In malignant human melanomas, a close correlation has been observed between tumor-associated macrophage infiltration and microvessel density (Ono et al., 1999). In a transgenic mouse model of squamous epithelial carcinogenesis, stromal infiltration of mast cells has been causally linked to the activation of angiogenesis (Coussens et al., 1999), and mast cell-deficient KIT^{W}/ KIT^{Wv} mice were shown to have decreased tumor-associated vascularization (Starkey et al., 1988). The angiogenic effect of the inflammatory cells has been attributed to their capacity to induce proangiogenic factors. For example, the macrophage-derived cytokine TNF-α can upregulate the expression of bFGF and VEGF, and IL-4, which is secreted by activated T lymphocytes, basophils, and mast cells, has morphogenic and mitogenic activity toward vascular endothelial cells (Klein et al., 1993; Ryuto et al., 1996). In addition, the induction of angiogenesis by inflammation has been functionally linked to the secretion of inflammatory cell-derived proteases, which leads to the remodeling of the extracellular matrix and the subsequent release of sequestered angiogenic factors (Coussens and Werb, 2002). In support of this concept, a study employing a transgenic mouse model for islet cell carcinoma has demonstrated that the secretion of MMP-9 by tumor infiltrating inflammatory cells leads to the proteolytic mobilization of VEGF (Bergers et al., 2000). Furthermore, experimentally induced squamous carcinogenesis is dependent on MMP-9 expressed by inflammatory cells (Coussens et al., 2000).

In contrast to progress made in understanding the functional relationship between tumor inflammation and angiogenesis, the signals that elicit tumor-associated inflammatory responses remain largely unidentified. CXCL-8 is a potent chemotactic factor for neutrophils and, as such, is intimately associated with the initiation of an inflammatory response (Matsushima et al., 1988; Mukaida et al., 1998). Therefore, the induction of CXCL-8 by oncogenic Ras could provide a mechanism by which neoplastic cells recruit immune cells to the tumor site to facilitate angiogenesis. This idea is supported by the functional relationship that we observe between CXCL-8-dependent tumor infiltration by inflammatory cells and the recruitment of endothelial cells. It should be noted that CXCL-8 might also contribute to the proangiogenic effects of oncogenic Ras through its ability to directly stimulate endothelial cell proliferation and chemotaxis (Koch et al., 1992). Therefore, CXCL-8 secretion by tumor cells expressing oncogenic Ras can be exploited in diverse ways to elicit angiogenic responses.

Clinical studies have demonstrated that CXCL-8 is upregulated in several human malignancies, including breast cancer (Yokoe et al., 1997), colon cancer (Cuenca et al., 1992), pancreatic cancer (Le et al., 2000), melanoma (Nurnberg et al., 1999; Singh et al., 1999), and non-small cell lung cancer (NSCLC) (Smith et al., 1994). Generally, CXCL-8 production is linked with tumor vascularization, metastatic phenotype, and overall poor prognosis (Ueda et al., 1994; Kitadai et al., 1998; Nurnberg et al., 1999; Singh et al., 1999). Moreover, the tumorigenic potential of human colon, pancreatic, and prostate cancer cells has been shown to directly correlate with the amount of CXCL-8 expression (Shi et al., 1999; Inoue et al., 2000; Li et al., 2001). We have found that the extent of CXCL-8 induction depends on the concentration of oncogenic Ras. Thus, while an appreciable increase in CXCL-8 production is detected in cells expressing near physiological levels of Ras, this response is significantly enhanced at supraphysiological levels. Since amplification of activated alleles of Ras genes has been shown to occur during progression of certain human malignancies (Thor et al., 1986; Suhardja et al., 2001; Heidenblad et al., 2002), the aggressive nature of these tumors might be dictated, at least in part, by the high levels of CXCL-8 expression.

We have found that full stimulation of CXCL-8 transcription by Ras requires the activation of both the ERK/MAPK pathway and PI-3 kinase. However, activation of the ERK cascade alone was sufficient to induce a partial response. Consistent with this observation, Raf activation was found to induce CXCL-8 expression in a global gene expression analysis using MCF-10A cells (Schulze et al., 2001). Activating mutations of BRaf are found with high frequency in human melanomas, and, in metastatic lesions, the presence of constitutively active BRaf is associated with poor prognosis (Houben et al., 2004). Notably, melanoma growth and metastasis is enhanced by tumor-derived CXCL-8 expression (Bar-Eli, 1999). Although the functional relevance of BRaf activation for CXCL-8 expression in human melanoma remains to be determined, the plausible cause-and-effect relationships between these two events suggests that deregulated activity of Ras effector pathways may contribute to tumor progression through the upregulation of CXCL-8.

Xenograft studies in immunocompromised nude mice have been used extensively to analyze cellular and molecular mechanisms of tumor angiogenesis. In the present study, we have utilized this experimental system to gain insight into the role of CXCL-8 in mediating Ras-induced tumorigenesis. Although nude mice are compromised in acquired immune responses due to the lack of mature T lymphocytes, innate immunity in these animals is largely intact. Since CXCL-8 primarily targets neutrophils, which act as initiators of acute inflammatory responses, the nude mouse provides an adequate context to analyze the significance of Ras-induced CXCL-8 expression in tumor inflammation and angiogenesis. Nevertheless, future studies using mouse models that more closely recapitulate the in vivo progression of spontaneously arising human tumors will be instrumental in strengthening the implications of our observations for the pathogenic mechanism of cancer development.

Experimental procedures

Cell culture

HeLa and H125 cells were maintained in Dulbeccos' modified Eagle medium (DMEM) supplemented with 10% fetal bovine serum (FBS) (Gibco BRL). MCF10A cells were grown in Ham's nutrient F12/DMEM (1:1) containing 5% horse serum, 10 μg/ml insulin, 20 ng/ml EGF, 5 μg/ml hydrocortisone, and 100 ng/ml cholera toxin. All cells were cultured at 37°C in the presence of 5% CO_2. To generate Tet-Off stable cell lines, tetracycline-transactivator (tTA)-transgenic HeLa cells (Clonetech) were cotransfected with a hygromycin-resistance marker and a plasmid encoding the constitutively active mutant (12G→V) of HA-epitope tagged H-RasV12 under a tetracycline-responsive promoter (pTRE-H-RasV12) and selected with 200 μg/ml hygromycin for two weeks. Resistant clones were isolated and tested for tetracycline-responsive expression of the transgene.

Western blot analysis

Cells were lysed and protein concentrations of cell lysates were determined by Coomassie protein assay (Pierce). Samples were separated on 12.5%

SDS-polyacrylamide gels and electrophoretically transferred to nitrocellulose membranes (Schleicher & Schuell). Blots were incubated with the following antibodies: Ras-specific rabbit polyclonal, HA-epitope specific mouse monoclonal (12CA5), or tubulin-specific mouse monoclonal (Sigma). Immune complexes were visualized by incubation with either a goat anti-rabbit or a goat anti-mouse HRP-conjugated secondary antibody (Cappel). The immunoreactive band was visualized by enhanced chemiluminescence (Perkin-Elmer).

RNA isolation and reverse transcription (RT)-PCR analysis

Total RNA was prepared using the RNeasy RNA isolation kit (Qiagen). cDNA was synthesized from 2.5 μg of total RNA using an Oligo-dT primer (Invitrogen) in a 20 μl reaction. 2 μl of cDNA was then used to amplify the human CXCL-8 sequence, the transgene-specific sequence of HA-H-RasV12 and HA-K-RasV12, the partial sequence of human CXCR-1 and -2, two splice variants of human VEGF-A, or human thrombospondin using specific PCR primers (see Supplemental Data at http://www.cancercell.org/cgi/content/full/6/4/447/DC1/). The conditions for all PCR reactions were: 3 min at 94°C, followed by 1 min at 95°C, 1 min at 55°C, 1 min at 72°C for 25 cycles, and then 1 cycle of 10 min at 72°C. PCR amplification of the 1007 bp sequence of human GAPDH was used to control the quality of the cDNA.

ELISA assay

Tissue culture supernatants or serum were analyzed in duplicate using human CXCL-8 specific ELISA purchased from R&D Systems.

Adenoviral infection

Replication-deficient recombinant adenovirus (Ad5ΔE1) driving expression of either β-galactosidase, HA-H-RasV12, or HA-K-RasV12 was produced in 293 cells. Target cells were infected with ~500 recombinant viral particles per cell. To assess adenoviral infection efficiency, cells infected with the Ad-β-galactosidase construct were fixed 24 hr after infection with 0.5% glutaraldehyde in PBS for 15 min and subsequently stained with X-Gal at 37°C to detect β-galactosidase-positive cells.

Luciferase and β-galactosidase assay

HeLa cells were plated at a density of 5×10^4 cells per well in a 24-well-plate. Luciferase-reporter plasmid (250 ng/well) was cotransfected with Tk-β-galactosidase (250 ng/well) plus effector plasmids (500 ng/well) using the FuGene 6 transfection Reagent (Roche). Empty vector (PCGT) was used to equalize the total amount of transfected DNA in all samples. Luciferase activities were measured 24 hr after transfection and normalized to β-galactosidase activities. Results are represented as mean values and SD from three independent experiments. Activity achieved by RasV12-expression is arbitrarily set as 100%.

In vivo tumor formation assays

Studies were conducted in eight-week-old immunocompromised athymic male mice (NIH Swiss Nude, Taconic Farms). 1×10^6 cells were resuspended in 150 μl sterile PBS and injected subcutaneously with a 26-gauge needle into the flank of anaesthetized animals. Tumor size was monitored every two days using calipers, and the length (l) and width (w) of the developing tumor was converted to volume using the equation $(w^2 \times l)/2$. Mice were sacrificed when tumors reached a maximum size of 2 cm in the largest diameter. For antibody injections, mice were randomized into two groups of six mice two days after tumor cell injection. The experimental group received CXCL-8 neutralizing monoclonal antibody (MAB208 [clone 6217] R&D Systems) or I8-6 (Antigenix America) (100 μg/mouse injected at the tumor site) on days 2, 6, 9, 13, and 16. Controls received 100 μg IgG_1 isotype-matched antibody (Rockland) per animal on the same days as the experimental group. Upon sacrifice, fragments of the tumor were fixed in 4% formalin for histological and immunohistochemical examinations. Remaining fragments were immediately snap-frozen in liquid nitrogen for either frozen sections or for protein and RNA extraction. All animal studies were approved by Institutional Animal Care and Use Committee (IACUC) protocols.

Granulocyte depletion in vivo

Mice were treated with a monoclonal antibody against Ly-6C/Gr-1 (RB6-8C5, BD Biosciences) 24 hr prior and 48 hr after tumor cell inoculation. The antibody was applied i.p. at a concentration of 100 μg/mouse per injection. Control animals received an identical dose of rat IgG_{2b} control antibody (clone A95-A, BD Biosciences).

Tumor morphological and histochemical examination

For immunohistochemical staining, formalin-fixed or snap-frozen fragments of tumor specimens were sectioned to 5 μm thickness. Paraffin-embedded sections were deparrafinized and rehydrated. Primary antibodies were added in the following dilutions: anti-PCNA 1:100 (Santa Cruz), anti-α_MIntegrin 1:100 (Santa Cruz), anti-CD31 1:50 (BD Biosciences). The sections were incubated overnight at 4°C, after which the appropriate biotinylated secondary antibody (Vector Laboratories) was added. Following secondary antibody incubation, the VectaStain Elite ABC Reagent (Vector Laboratories) was applied, and the subsequent antibody/enzyme conjugate was developed with DAB. All sections were counterstained with hematoxylin. Hematoxylin and eosin staining was performed separately by the University Histology Services (SUNY Stony Brook).

Quantitation of tumor composition and vascular density

Sections from peripheral and central regions of a tumor were imaged at low magnification (2.5×), and areas of viable tumor tissue and necrosis were evaluated using the Zeiss Axiovision software program. Blood vessel density was quantified by counting the total number of CD31-positive vessels across the whole section of tumors. The diameter of blood vessels was calculated with the Zeiss Axiovision software program. For these analyses, six different tumor samples were evaluated per experimental condition.

Flow cytometry

HTO cells were induced for transgene expression 24 hr prior to the experiment. Cells were incubated with FITC-conjugated monoclonal antibodies to CXCR-1 or CXCR-2 (BD Biosciences) or FITC-labeled matched mouse IgG isotype (Rockland) as a control for 2 hr at 4°C. Fluorescence was measured in a Becton Dickinson FACScalibur.

Reagents and mammalian expression plasmids

The following pharmacological inhibitors at indicated working concentrations were employed in our studies: 10 μM MEK inhibitor U0126 (CalBiochem); 20 μM PI3K inhibitor LY 294002 (CalBiochem). Recombinant human CXCL-8 was purchased from R&D Systems. pCGT was used as a mammalian expression vector to express RasV12, RafCAAX, RacV12, and RacN17. The following constructs were generously provided: MKK7-JNK1 by Dr. Roger Davis (University of Massachusetts, Worcester), myr-Akt by Dr. Nissim Hay (University of Chicago), p110CAAX by Dr. Julian Downward (London Research Institute), pCMV4-IκBαS32A/S36A by Dr. Nancy Reich (SUNY Stony Brook), and the CXCL-8 luciferase reporter construct by Dr. Erich Mackow (SUNY Stony Brook).

Statistical analysis

Statistical significance for in vivo experiments was determined by one-way analysis of variance (ANOVA) with the Tukey-Kramer multiple-comparison post-test (GraphPad Instat, GraphPad Software Inc.). Comparison between two groups was made using Student's t test for unpaired data when appropriate. In all analysis, $p < 0.05$ was considered statistically significant.

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