Published in final edited form as:

Mol Interv. 2008 February ; 8(1): 22–27. doi:10.1124/mi.8.1.6.

Oncogenic Ras–Induced Expression of Cytokines in Cancer

Brooke B. Ancrile, Kevin M. O’Hayer, and Christopher M. Counter

Department of Pharmacology and Cancer Biology, Duke University Medical Center, Durham, North Carolina 27710, USA.

Abstract

The Ras family of small guanosine triphosphatases normally transmit signals from cell surface receptors to the interior of the cell. Stimulation of cell surface receptors leads to the activation of guanine exchange factors, which, in turn, convert Ras from an inactive GDP-bound state to an active GTP-bound state. However, in one third of human cancers Ras is mutated to become oncogenic, and remain in the constitutively active GTP-bound state. In this oncogenic state, Ras activates a constellation of signaling that is known to promote tumorigenesis. One consequence of this oncogenic Ras signal in cancer cells is the upregulation of the cytokines interleukin (IL)-6, IL-8, and chemokine growth-regulated oncogene (GRO). We review the evidence supporting a role for these cytokines in Ras-driven directed solid tumors.

Ras and Cancer

The Ras family of small guanosine triphosphatases (GTPases), composed of N-, H- and K-Ras, normally transmit signals from cell surface receptors to the interior of the cell. Stimulation of cell surface receptors leads to the activation of guanine exchange factors (GEFs), which, in turn, convert Ras from an inactive GDP-bound state to an active GTP-bound state. In this active state, Ras adopts a conformation that permits effector proteins such as phosphoinositide 3-kinases (PI3Ks), the Rafs mitogen-activated protein kinase kinase kinases, and RalGEFs to bind Ras, leading to their activation and propagation of signaling. This signal is terminated by GTPase-activating proteins (GAPs) that stimulate the GTPase activity of Ras, returning Ras to its inactive GDP-bound state (1).

In most cancers, Ras is inappropriately activated. In fact, 20–30% of all tumors harbor oncogenic point mutations in Ras at Gly¹², Gly¹³, or Gln⁶¹ that impair the intrinsic and GAP-stimulated GTP hydrolysis, leaving oncogenic Ras in a constitutively active GTP-bound state (2). In pancreatic cancer, up to 90% of tumors carry an activating Ras mutation (2). If Ras itself is not mutated, often Ras signaling pathway components are inappropriately activated or repressed to promote Ras signaling. Specifically, upstream cell surface receptors that activate Ras, such as epidermal growth factor receptor (EGFR) and the closely related Her2/Neu, are often amplified or mutated to remain active, resulting in chronic Ras activation (1). In fewer cases, there is a loss of a RasGAP, keeping Ras in an active, GTP-bound state (1). Additionally, downstream activating mutations in Raf and PI3K or loss of the lipid phosphatase *PTEN* leads to activation of individual Ras effector pathways (1). Thus, the Ras pathway is inappropriately activated in most cancers.

A plethora of data demonstrates that constitutive activation of Ras promotes tumor phenotypes. Gain-of-function mutations to Ras that occur in human cancers endow the protein with the ability to promote cell proliferation, cell survival, cell migration, tumor growth, and metastasis in cell culture and animal models (3). In contrast, extinguishing oncogenic Ras expression in established tumors leads to tumor regression (4). Thus, understanding how Ras promotes tumorigenesis could have therapeutic value in the treatment of many types of cancers.

Oncogenic Ras and Cytokines

Activation of Ras induces an autocrine signal in tumor cells that promotes many tumor cell phenotypes (1), but it is now appreciated that Ras activation in tumor cells also leads to paracrine signaling to the tumor microenvironment (5–7). The tumor microenvironment plays a critical role in tumorigenesis (5–7). Modification of the tumor stroma, which is composed of fibroblasts, immune and inflammatory cells, adipocytes, and endothelial cells, is important for tumor growth and development. During tumor initiation, paracrine signaling from cancer cells modulates the surrounding stroma to establish a microenvironment conducive for tumor growth (7). Important modulations of the stroma include activation of fibroblasts and recruitment of inflammatory cells, which secrete proteases that release growth factors sequestered in the extracellular matrix (6,7). The recruitment of endothelial cells for the formation of a vascular system, which provides oxygen and nutrients to the tumor cells (5), is also an important step in the establishment of a microenvironment. Infiltrating inflammatory cells in the tumor stroma also sustain and promote neovascularization (8). An increasing number of examples demonstrate that activation of Ras in tumor cells positively acts to foster changes in the tumor microenvironment. For example, oncogenic Ras has been shown to promote tumor cell invasion, in part due to the induction of matrix metalloproteinases that degrade the extracellular matrix, allowing tumor cells to escape and metastasize (9). Oncogenic Ras has also been linked to the induction of tumor angiogenesis. Loss of oncogenic Ras expression in tumor cells results in apoptosis of cells expressing CD31, a marker for endothelial cells, and a subsequent collapse in tumor vasculature and regression of the tumor, strongly suggesting that oncogenic Ras paracrine signaling promotes survival of endothelial cells (10). Additionally, oncogenic Ras has been shown to transcriptionally upregulate vascular endothelial growth factor (VEGF), which acts upon endothelial cells to promotes angiogenesis (11).

It has recently come to light that one class of secreted proteins that could mediate oncogenic Ras paracrine signaling is cytokines. Cytokines are a class of small secreted proteins which act in a paracrine manner to facilitate the interaction and communication of other cells, often eliciting an immune response. New findings reviewed here indicate that oncogenic Ras upregulates the expression of the cytokines interleukin (IL)-8 (also termed CXCL-8), IL-6, and chemokine growth-regulated oncogene 1 (GRO-1).

Sparmann and Bar-Sagi (12) reported that activation of a tetracycline (Tet)-inducible H-Ras^{G12V} transgene in the cancer cell line HeLa led to elevated mRNA and secreted protein levels of IL-8, a member of the Glu-Leu-Arg N-terminal sequence-containing (ELR⁺) CXC family of cytokines. Overexpression of oncogenic H-Ras similarly increased expression and secretion of IL-8 in lung carcinoma (H125) and breast epithelial (MCF10A) cell lines, suggesting that this effect was specific to one cell type. Increased IL-8 expression was critical for tumor growth in the H-Ras^{G12V}-expressing HeLa cells, as inhibition of IL-8 activity (via injection at the tumor site of an IL-8 neutralizing antibody twice weekly for sixteen days) reduced the subcutaneous tumor growth in immunocompromised mice by ~60% (Figure 1). Mechanistically, the tumor-promoting activity of secreted IL-8 was attributed to enhancing angiogenesis through paracrine signaling. This supposition is supported by the following observations. First, the tested HeLa cells lacked expression of the IL-8 receptors, CXCR-1 and -2. Second, inhibition of IL-8 via injection of the aforementioned IL-8 neutralizing antibody led to a >80% decrease in the number of inflammatory cells within the tumor. Consistent with the link of inflammatory cells to angiogenesis, inhibition of IL-8 decreased CD31⁺ (endothelial) cells in early stage tumors by approximately 50% without any detectable change in cell proliferation (PCNA staining) or apoptosis (TUNEL staining). End stage tumors exhibited necrosis in two-thirds of the tumor volume, consistent with the diminished vasculature of the tumors. Taken together, these data suggest that oncogenic H-Ras-induced IL-8 expression in tumor cells plays an important role in tumor development by promoting the

inflammatory response and development of tumor angiogenesis. It is worth noting that IL-8 concentrations are elevated in the serum of cancer patients, including those with pancreatic (13), lung (14), melanoma (15), breast (16,17), prostate (18), and ovarian cancers (19). Moreover, in breast cancer elevated levels of circulating IL-8 correlate with advanced disease and diminished survival rate (16).

The aforementioned studies demonstrate the essential role of H-Ras-induced IL-8 secretion in tumorigenesis of human cancer cell lines engineered to overexpress oncogenic H-Ras when injected into immunocompromised mice. Wislez et al. (20) expanded these results to a more physiologically relevant K-Ras-driven transgenic mouse lung tumor model in which lung tumors were driven by the expression of endogenous levels of oncogenic KRAS, the most commonly mutated Ras family member, in immunocompetent mice. Specifically, K-Ras^{LA1} mice, engineered to contain an oncogenic (G12D) version of the KRAS transgene inserted in the KRAS locus, develop K-Ras^{G12D}-driven lung adenocarcinomas. Using this mouse model of lung cancer, elevated expression of macrophage inflammatory protein-2 (MIP-2) and keratinocyte chemoattractant (KC), the mouse functional homologs of IL-8, were detected in lung tissue homogenates of mice harboring the activated KRAS allele compared to wild type littermates, confirming that, in this more physiological setting, oncogenic KRAS induces secretion of mouse IL-8 homologs. Moreover, thrice weekly intraperitoneal injections for three weeks of a neutralizing antibody serum to CXCR2, a receptor for the ELR⁺ CXC family of cytokines that includes MIP-2 and KC, reduced the number of lesions in the lungs of mice by ~30% and the development of more malignant lesions compared to control mice injected with a control antibody (Figure 1). Mechanistically, K-Ras^{G12D}-mediated secretion of MIP2 and KC was again attributed to paracrine signaling. First, despite the obvious effect *in vivo* of the CXCR2 neutralizing antibody serum on tumor growth, the same antibody did not affect the *in vitro* growth of LKR-13 cells, a lung adenocarcinoma line isolated from this mouse background. Second, progression of lung tumorigenesis correlated with the recruitment of CXCR2-expressing neutrophils and endothelial cells to these lesions. Third, pre-incubation with the CXCR2 neutralizing antibody serum inhibited the recruitment of alveolar inflammatory cells by LKR-13 media in a migration assay. Overall, these findings support a model whereby oncogenic K-Ras-mediated secretion of IL-8 acts in a paracrine fashion to promote tumorigenesis.

In addition to IL-8, another cytokine in the same ELR⁺ CXC family, GRO-1, has been linked to the Ras-mediated modulation of the tumor microenvironment. Specifically, human ovarian epithelial cells that ectopically express both the catalytic subunit of telomerase (hTERT) and the SV40 viral large T and small t (T/t) antigens were found to express GRO-1 and other cytokines upon expression of H-Ras^{G12V} or K-Ras^{G12V} (21). Yang et al. (22) found that short hairpin (sh)RNA-mediated knockdown of GRO-1 expression in such H-Ras^{G12V}-expressing ovarian cells resulted in a >70% reduction in tumor size when grown in immunocompromised mice (Figure 1). In this case, however, the paracrine effect was not linked to inflammatory cells, but rather the fibroblast component of the tumor stroma. Specifically, GRO-1 treatment of cultured fibroblast with recombinant GRO-1 increased the number of β -gal positive cells, a marker of the growth arrest state of senescence. Moreover, mixing GRO-1-treated fibroblasts with an otherwise immortalized but non-transformed ovarian epithelial line enabled tumor formation when injected into immunocompromised mice. Lastly, upon examination of normal and cancerous human ovarian tissue samples, an increase in the number of β -gal⁺ fibroblasts in tumor tissues as compared to normal control tissues was noted, and that fibroblast cell lines isolated from these tumor tissues expressed higher levels of GRO-1 compared to normal ovarian fibroblast lines. These results suggest that GRO-1 participates in Ras-induced tumorigenesis via the modulation of tumor-associated fibroblasts.

Our own studies examined the role of IL-6 in oncogenic Ras-mediated cancer (23). These studies were prompted by the observation that IL-6 levels are elevated in the serum of patients diagnosed with pancreatic, lung, colorectal, melanoma, breast, prostate, and ovarian cancers (24), cancers that are associated with either mutations in the Ras oncogene itself or mutations in the Ras signaling pathway (2). Moreover, IL-6 contributes to several cancer phenotypes, including cell survival (25), tumor angiogenesis (26), and, possibly, tumor metastasis (27), and is often associated with poor outcome and disease progression. Specifically, in human embryonic kidney (HEK) cells stably expressing hTERT and SV40 T/t antigens, secreted IL-6 concentrations were elevated, and cells became tumorigenic upon the tamoxifen-induced expression of an ER:Ras^{G12V} fusion protein, as compared to uninduced, nontumorigenic cells. These results suggested that IL-6 induction correlated with the tumorigenic activity of oncogenic Ras. A similar increase in IL-6 mRNA and protein was detected in tumorigenic fibroblasts, myoblasts, and mammary epithelial cells expressing SV40 T/t antigens, hTERT, and H-Ras^{G12V} as compared to the isogenic non-tumorigenic counterparts lacking H-Ras^{G12V}. Using three independent shRNAs, stable knockdown of IL-6 reduced tumor size of the aforementioned Ras^{G12V}-transformed HEK cells compared to scramble control when injected into immunocompromised mice (Figure 1). Moreover, the same feat was achieved by injecting mice harboring such tumors with a human IL-6-specific neutralizing antibody, which, like shRNA, only neutralizes tumor-derived IL-6 (Figure 1). As with the detection of elevated IL-6 upon induction of the tumorigenic state by oncogenic Ras in different cell types, knockdown of IL-6 via shRNA similarly reduced tumorigenic growth of Ras-transformed myoblasts and fibroblasts. These results were validated in *IL-6*^{-/-} mice. Specifically, *IL-6*^{-/-} mice treated topically with the tumor initiator 7,12-dimethylbenz[a]anthracene (DMBA) and the tumor promoter tetradecanoyl phorbol acetate (TPA), which are known to induce skin papillomas characterized by oncogenic Ras mutations (28), exhibited a thirtyfold reduction in the total tumor burden, as compared to isogenic *IL-6*^{+/+} control mice (Figure 1). Moreover, knockdown of IL-6 in pancreatic cancer cell lines characterized by K-Ras oncogenic mutations also reduced their tumorigenic capacity when injected into immunocompromised mice. Mechanistically, oncogenic Ras-induced secretion of IL-6 appeared to promote tumor growth via paracrine signaling. First, cells in which IL-6 shRNA inhibited tumor growth lacked detectable IL-6 receptor. Second, shRNA-mediated reduction of IL-6 in these Ras-transformed cells did not inhibit their growth in tissue culture. Third, immunohistochemical analysis of tumors resulting from Ras-transformed HEK cells showed that knockdown of IL-6 resulted in a 95% decrease in the number of endothelial (CD31⁺) cells in the tumor. As seen with the ELR⁺ CXC cytokines IL-8 and GRO-1, secretion of the cytokine IL-6 is also induced by expression of oncogenic Ras in tumor cells, and this secretion promotes tumorigenesis in a paracrine fashion.

Targeting Ras-Induced Cytokines

Multiple models demonstrate that inhibiting the chronic activation of Ras, resulting from either activating mutations in Ras or illegitimate activation of cell surface receptors, greatly impedes tumor growth. Translating this to the clinic, however, has been difficult. For example, Ras must be posttranslationally modified by the addition of lipid moieties for oncogenic activity (29), yet small-molecule inhibitors that specifically interfere with the enzyme adding one such lipid moiety, farnesyl transferase, have failed to demonstrate Ras-specific anti-tumor activity in the clinic. Moreover, these inhibitors do not inhibit K- or N-Ras membrane targeting, because these Ras family members can acquire a different membrane-anchoring lipid moiety through the activity of geranyl-geranyl transferase (30). Unfortunately, the use of inhibitors against geranyl-geranyl transferase or both geranyl-geranyl and farnesyl transferases is hampered in the clinic by toxicity (31).

Given the difficulty of directly inhibiting Ras, inhibition of Ras signaling has been pursued. The Raf inhibitor BAY 43-9006 has some efficacy in colon, breast, and non-small-cell lung carcinomas(32), and small-molecule inhibitors to the downstream targets of Rafs, and MEK1 and MEK2, have yielded positive results in the clinic in the treatment of renal cell carcinoma (32). Inhibition of the PI3K pathway via mTOR inhibitors has also yielded limited results (32). Thus, the concept of targeting Ras signaling has merit as a means to inhibit the growth of Ras-driven tumors. In this regard, cytokines also represent an attractive target for Ras signaling as: 1) oncogenic Ras induces the secretion of cytokines; 2) IL-6 and IL-8, as well as other cytokines, are elevated in the serum of patients diagnosed with cancers characterized by oncogenic Ras mutations (13–15,17–19,24); 3) genetic ablation, shRNA knockdown, or neutralizing antibodies against such cytokines inhibit oncogenic Ras-mediated tumor growth (12,20,22,23); 4) inhibition of cytokines may be tolerated in patients, at least in the case of IL-6, as genetic ablation of IL-6 in mice is not lethal (33); 5) cytokines are located in the interstitial space and as such are much more bioavailable than intracellular targets; and 6) antibodies that are already available (or could be generated) can be used to inhibit cytokine function, with the added advantages that antibody therapy is generally well tolerated in patients (34)—preliminary studies already demonstrate anti-tumor activity of IL-8 and IL-6 neutralizing antibodies (12,23). We therefore suggest that humanized antibodies against cytokines like IL-8, GRO-1, and IL-6 may have utility in the treatment of Ras-driven cancers (Figure 2). Indeed, inhibition of proteins by humanized antibodies has proven successful in the clinic, as exemplified by bevacizumab (Avastin®), as well as a variety of other anti-VEGF drugs (35). Several completed and ongoing clinical trials have investigated the ability of monoclonal antibodies to inhibit IL-6 function for cancer therapeutics, typically in multiple myeloma that depend upon IL-6 (24), but additional trials are focusing on the treatment of prostate cancer with this monoclonal antibody (36). In conclusion, the discovery that oncogenic Ras increases the secretion of cytokines that are important for tumor growth may provide a way to target a pathway activated in the vast majority of cancers.

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Biographies

Brooke B. Ancrile

Brooke is a graduate student at Duke University in the University Program in Genetics and Genomics. Her research focuses on the role of cytokines in Ras-induced tumorigenesis.

Kevin M. O'Hayer

Kevin is a graduate student at Duke University in the Department of Pharmacology and Cancer Biology. His research focuses on the role of chemokines in Ras-induced tumorigenesis.

Christopher M. Counter.

Chris Counter is an Associate Professor at Duke University in the Department of Pharmacology and Cancer Biology, and the Department of Radiation Oncology. His research focuses on cell transformation and immortalization.

Figure 1.

Inhibiting cytokine function impedes oncogenic Ras-driven tumor growth. Oncogenic Ras-mediated activation of cytokine gene transcription (double helix) in tumor cells (brown oval) leading to increased cytokine mRNA (purple wavy line) and protein (blue spheres) can be blocked by A) knock out of the cytokine gene, B) RNAi targeting the cytokine mRNA or C) cytokine neutralizing antibodies inhibits tumor growth.

Figure 2.

Oncogenic Ras stimulated cytokine secretion promotes tumor growth. Oncogenic Ras (Ras-GTP) promotes the transcription of cytokine genes (double helix), leading to elevated levels of secreted cytokines (blue spheres) that act to modulate the immune system (yellow cells), promote angiogenesis (purple capillaries) and activate tumor stroma (blue cells).