Identification of an Autoregulatory Feedback Pathway Involving Interleukin-1α in Induction of Constitutive NF-κB Activation in Pancreatic Cancer Cells*

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We recently reported that NF-κB is constitutively activated in most human pancreatic cancer tissues and cell lines but not in normal pancreatic tissues and immortalized pancreatic ductal epithelial cells. IκBα-mediated inhibition of constitutive NF-κB activity in human pancreatic cancer cells suppressed tumorigenesis and liver metastasis in an orthotopic nude mouse model, suggesting that constitutive NF-κB activation plays an important role in pancreatic tumor progression and metastasis. However, the underlying mechanism by which NF-κB is activated in pancreatic cancer remains to be elucidated. In this study, we found that an autocrine mechanism accounts for the constitutive activation of NF-κB in metastatic human pancreatic cancer cell lines. Further investigation showed that interleukin-1α was the primary cytokine secreted by these cells that activates NF-κB. Neutralization of interleukin-1α activity suppressed the constitutive activation of NF-κB and the expression of its downstream target gene, urokinase-type plasminogen activator, in metastatic pancreatic cancer cell lines. Our results demonstrate that regulation of interleukin-1α expression is primarily dependent on AP-1 activity, which is in part induced by signaling pathways that are epidermal growth factor receptor-dependent and -independent. In conclusion, our findings suggest a possible mechanism for the constitutive activation of NF-κB in metastatic human pancreatic cancer cells and a possible missing mechanistic link between inflammation and cancer.

NF-κB is a family of pleiotropic transcription factors that control the expression of numerous genes involved in growth, tumorigenesis, tumor metastasis, differentiation, embryonic development, apoptosis, and inflammation (1–4). Five members of mammalian NF-κB have been described: NF-κB1 (p50 and its precursor p105), NF-κB2 (p52 and its precursor p100), c-Rel, RelA (p65), and RelB, each of which has a 300-residue-long Rel homology domain (2, 3, 5). The interaction of c-Rel, RelA, and RelB with their inhibitors, the IκBs, results in inactive complexes in the cytoplasm by masking the nuclear localization signal (6, 7). The inhibitor proteins IκBo, IκBβ, IκBγ, IκBε, and Bcl-2 and the Drosophila protein Cactus have been described and characterized (2, 3, 5). In most cell types, NF-κB proteins are sequestered in the cytoplasm by the inhibitor IκB in an inactive form (2, 6, 7). On stimulation, IκB is phosphorylated by IκB kinase, which triggers its rapid degradation (8–10). Consequently NF-κB proteins are released and translocated into the nucleus where they activate the expression of target genes (1–3, 5). One of the key target genes regulated by NF-κB is its inhibitor IκBo. A feedback inhibition pathway for control of IκBo gene transcription and down-regulation of transient activation of NF-κB activity has been described (11–13).

Members of the NF-κB family are involved in the development of cancer. For instance, v-Rel, which is carried by a highly oncogenic retrovirus, causes an aggressive tumor in young birds and is able to transform avian lymphoid cells and fibroblasts (1, 14). The mutated c-rel oncogene also transforms cells. Many reports have demonstrated that chromosomal amplification, overexpression, and recurrent genomic rearrangement in the genes encoding c-Rel, Bcl-3, p105 (p50), and p100 (p52) have been identified in many human hematopoietic cancers and several types of solid tumor such as human non-small cell lung carcinomas, squamous cell carcinomas of the head and neck, and adenocarcinomas of breast (1, 15). Many tumors have acquired genetic alterations in the signaling pathways that regulate NF-κB activation. For example, defective IκBo led to constitutive nuclear NF-κB activity, which in turn conferred a growth advantage of Hodgkin’s disease tumor cells (16). The elevated IκB kinase activities were also found in some of the tumor cells, suggesting that IκB kinase was activated by as yet unidentified aberrant upstream signaling cascades (16). We previously reported that RelA, the p65 subunit of the NF-κB transcription factor, was constitutively activated in most pancreatic cancer tissues and human pancreatic cancer cell lines but not in normal pancreatic tissues and immortalized pancreatic ductal epithelial cells (17, 18). Our recent work showed that inhibition of constitutive NF-κB activity by a mutant IκBo (S32A,S36A) completely suppressed the liver metastasis of the pancreatic cancer cell line ASPC-1 and the tumorigenic phenotype of a nonmetastatic pancreatic tumor cell line, PANC-1, suggesting that constitutive RelA activity plays a key role in pancreatic cancer metastasis and tumor progression (19, 20). However, the mechanisms by which NF-κB transcription factors are constitutively activated in pancreatic cancer still remain to be elucidated.

Several studies have shown that secretion of proinflammatory cytokines are increased in cancers (21–24). These proinflammatory cytokines include tumor necrosis factor-α, interferon-γ, interleukin-1, and interleukin-6. Interleukin-1α (IL-1α) is a cytokine produced by a variety of cells such as macrophages, fibroblasts, and epithelial cells and a possible missing mechanistic link between inflammation and cancer.
Inflammatory cytokines, such as tumor necrosis factor α (TNFα) and interleukin-1 (IL-1), are the potent activators of NF-κB that induce phosphorylation and rapid degradation of IκB, exposing the nuclear localization sequence of NF-κB and resulting in NF-κB nuclear translocation (25, 26). Some of these cytokines, such as IL-1 and TNF, have been reported to be regulated by NF-κB in some cancer cell lines (27–30). Therefore, it is possible that autocrine secretion of these cytokines forms a positive feedback loop that induces the constitutive NF-κB activation. The involvement of autocrine stimulation mechanisms of these cytokines in cancer progression has been suggested (31–35). For example, overexpression of IL-1 as an autocrine growth factor has been reported in a number of tumors in which activation of Raf plays an important role in transformation, suggesting that blockade of IL-1 signaling may be an approach to limiting the growth of certain tumors (31). Both exogenous IL-1α and endogenous IL-1α contribute to the
transcriptional activation of NF-κB and AP-1, induce the expression of IL-8, and promote cell survival and the growth of head and neck squamous cell carcinoma cell lines in vitro (32–34). A recent study also suggested that overexpression of IL-1 might confer the chemoresistance in certain pancreatic cancer cell lines through NF-κB activation (35). However, the mechanism by which the regulation of the expression of these inflammatory cytokines is altered is unclear. Whether the overexpression of inflammatory cytokines such as TNF and IL-1 activate NF-κB or the constitutive activity of NF-κB induces the overexpression of these cytokines in cancer cells is not known.

To determine the mechanisms by which NF-κB is constitutively activated in metastatic pancreatic cancer cells, we sought to delineate the signaling cascades that lead to constitutive NF-κB activation. We generated a number of pancreatic cancer cell lines that express a phosphorylation-defective IκBα mutant to inhibit constitutive NF-κB activity. Our results show that overexpression of IL-1α is primarily induced by AP-1 activity, which is partially dependent on the overexpression of epidermal growth factor receptor (EGFR) in several metastatic pancreatic cancer cell lines but not in nonmetastatic pancreatic cancer cell lines. Thus, autocrine stimulation of IL-1α, but not IL-1β, induces the constitutive activation of NF-κB, which in turn induces the expression of its downstream target genes.

**EXPERIMENTAL PROCEDURES**

**Cell Lines and Reagents**—The human pancreatic cancer cell lines ASPC-1, PANC-1, Capan-1, and CFPAC-1 were purchased from the American Type Culture Collection (ATCC) (Manassas, VA). Human pancreatic cancer cell lines MDAPanc-3 and MDAPanc-28 were established by Marsha Frazier and Douglas B. Evans (The University of Texas M. D. Anderson Cancer Center) (36, 37). The human pancreatic cancer cell lines ASPC-1/IκBαBM and MDAPanc-28/IκBαBM were constructed as described previously by Dong et al. (18). Wild-type mouse embryonic fibroblast (WT-MEF) cells were established in our laboratory according to the report by Beg et al. (38). All cell lines were grown in the original cell culture medium specified by the American Type Culture Collection and Dulbecco’s modified Eagle’s medium containing 10% fetal calf serum. Neutralizing IL-1α, IL-1β, TNFα, TNFβ, and IL-1 receptor type I antibodies were obtained from R&D Systems (Minneapolis, MN). EGFR neutralizing antibody C225 was obtained from Dr. Zhen Fan (The University of Texas M. D. Anderson Cancer Center).

**The Preparation of Conditioned Medium**—Human pancreatic cancer cells were cultured in serum-free medium for 48 h. The medium was then harvested and centrifuged at 1000 rpm for 5 min. Four milliliters
the primers used for IL-1β were 5′-AAGCTGTTGCTCAAAAGCC-3′ and 5′-TCTGGTACCTACTAGAAGCCAG-3′. The PC products were 419 bp for IL-1α and 498 bp for IL-1β. The PCR conditions were as follows: 94°C for 5 min and then 30 cycles performed at 94°C for 1 min, 55°C for 1 min, and 72°C for 1 min. Finally, samples were extended at 72°C for 7 min. The PCR products were cloned into a pCRII-TOPO vector (Invitrogen) and were subsequently sequenced to confirm their identity. The IL-1α (BamHI- and EcoRI-digested) and IL-1β (EcoRI-digested) cDNAs were purified and used as the probes for Northern blot analysis.

Total RNA (30 μg) was subjected to electrophoresis through a 1.2% agarose gel containing formaldehyde, transferred to a Hybond nylon filter (Osmonics, Westboro, MA), and ultraviolet cross-linked. The blots were hybridized with 32P-labeled human IL-1α or IL-1β cDNA and exposed to display the results.

**Primer Extension and DNA Sequencing**—Total RNA (25 μg) from ASPC-1 and MDAPanc-28 cells was preheated at 65°C for 5 min and then put on ice for 5 min. After that, 200 ng of 32P-end-labeled primer (5′-GCTGT AGTTG TGTTC TGGCT GA-3′), 5 units of RNase inhibitor, 2.5 μl of ImProm-II reverse transcriptase, and 1× reverse transcription reaction buffer (Promega, Madison, WI) were added. The mixture was incubated at 37°C for 15 min and then transferred to 42°C for 1 h. The reaction products were treated with RNase H for 30 min, boiled for 5 min, chilled on ice, and analyzed in 8% denaturing polyacrylamide gels with DNA sequencing reaction products of human IL-1α genomic clone using the same 32P-labeled primer and DNA size marker x174 (32P-end-labeled). DNA sequencing was performed using a Sequenase kit (United States Biochemical, Cleveland, OH).

**Mutagenesis of AP-1 Sites in IL-1α Promoter**—IL-1α promoter was cloned by PCR. The right primer was 5′-CGCTGCTAGTTGCTGTCGTCGTTCAATGCA-3′ (the sequence underlined is the restriction site for BamHI) and the left primer was 5′-CGGAGCTCTGGGAACCCAAAAAGGCTGAGGCCAT-3′ (the sequence underlined is the restriction site for SacI).

PCR products were cloned into the pGGL2 firefly luciferase vector (Promega). The mutagenesis was performed using the mutagenesis kit from Stratagene (La Jolla, CA). The primer used for AP1-1 (site 1) was 5′-CCCTC TTCAAAATTTTTCGTCGTTCAATGCA-3′, and the primer used for AP1-2 (site 2) was 5′-CCCTC TTCAAAATTTTTCGTCGTTCAATGCA-3′. The PCR products were 419 bp for IL-1α and 498 bp for IL-1β. The PCR conditions were as follows: 94°C for 5 min and then 30 cycles performed at 94°C for 1 min, 55°C for 1 min, and 72°C for 1 min. Finally, samples were extended at 72°C for 7 min. The PCR products were cloned into a pCRII-TOPO vector (Invitrogen) and were subsequently sequenced to confirm their identity.

The IL-1α (BamHI- and EcoRI-digested) and IL-1β (EcoRI-digested) cDNAs were purified and used as the probes for Northern blot analysis.

RESULTS

**Conditioned Medium from Metastatic Human Pancreatic Cancer Cells Induces NF-κB Activation**—We previously reported that NF-κB was constitutively activated in most human pancreatic cancer tissues and cell lines (17) and that inhibition of constitutive NF-κB activation suppresses tumorigenesis and liver metastasis of pancreatic cancer cell lines (19, 20). Constitutive NF-κB activity was greatly reduced in the metastatic pancreatic cancer cell lines when the fresh medium was replenished 1–2 h before the EMSA. One possible mechanism is that the constitutive NF-κB activity was induced through autocrine growth factors or by the microenvironment created by autocrine growth factors.
stimulation. To test whether human pancreatic cancer cells secrete an inducer for NF-κB activation, we cultured the human metastatic pancreatic cancer cell lines ASPC-1 and MDAPanc-28 and the nonmetastatic pancreatic cancer cell line PANC-1 in serum-free medium for 48 h and then isolated the conditioned media to stimulate WT-MEF cells at different times. Our results showed that the conditioned medium from ASPC-1 and MDAPanc-28 pancreatic cancer cell lines induced NF-κB activation in a time-dependent mode but did not increase Oct-1 DNA binding activity (Fig. 1A). TNFα induced NF-κB activation in WT-MEF cells, but the conditioned medium from PANC-1 cells did not (Fig. 1B). These results suggest that metastatic but not nonmetastatic human pancreatic cancer cell lines secrete the activators of NF-κB, which may in turn induce NF-κB constitutive activity in these cells. The competition and supershift assays confirmed the specificity and identity of the NF-κB DNA binding activity as p65(RelA)/p50 heterodimers (Fig. 1C). Conditioned medium-stimulated NF-κB activation was confirmed by the conditioned medium-induced and time-dependent degradation of IκBα (Fig. 1D). Furthermore the conditioned media from the other human metastatic pancreatic cancer cell lines, Capan-1, CFPAC-1, and MDAPanc-3, also induced NF-κB DNA binding activity in WT-MEF cells (data not shown). These results suggest that the secretion of NF-κB inducer is a common phenomenon in metastatic pancreatic cancer cells.

Interleukin-1α Is the Primary Cytokine Secreted by Human Metastatic Pancreatic Cancer Cell Lines—To identify which inducer is secreted by these pancreatic cancer cell lines, we first screened a panel of neutralizing antibodies of common inducers of NF-κB, including IL-1α, IL-1β, TNFα, and TNFβ, before we performed biochemical purification and identification approaches. The conditioned media from these cell lines were isolated and incubated with anti-IL-1α, anti-IL-1β, anti-TNFα, or -TNFβ neutralizing antibodies (2 μg/ml for 2 h) for 30 min as indicated. The Oct-1 DNA binding activities were determined as loading controls. CM, conditioned medium.
ity in the ASPC-1- and MDAPanc-28-conditioned media, but the other neutralizing antibodies did not have much effect (Fig. 2A). Consistently IL-1α neutralizing antibody blocked the NF-κB-inducing activity from the conditioned media of other human metastatic pancreatic cancer cell lines, Capan-1, CF-PAC-1, and MDAPanc-3 (data not shown). This implies that IL-1α is the major cytokine and NF-κB-inducing activity secreted by these cells.

To determine whether IL-1α autocrine stimulation induces constitutive NF-κB activity in ASPC-1 and MDAPanc-28 cells, we treated these cells directly with neutralizing antibodies against IL-1α, IL-1β, TNFα, and TNFβ for 1 h and isolated the nuclear extracts for EMSA. Anti-IL-1α neutralizing antibody completely inhibited constitutive NF-κB activity in these cells, but anti-IL-β, -TNFα, and -TNFβ neutralizing antibodies only had minimal effects on constitutive NF-κB activity (Fig. 2B). To provide further evidence of the role of IL-α in autocrine stimulation, we treated WT-MEFs and MDAPanc-28 and ASPC-1 cells with anti-IL-1 receptor type 1 antibodies for 120 min to block their IL-1 receptors. The results showed that blocking the IL-1 receptor in these cells inhibited IL-1α-induced NF-κB activation (Fig. 2, C and D). Together these results suggest that IL-1α is the principal cytokine that contributes to the induction of constitutive NF-κB activity in MDAPanc-28 and ASPC-1 cells.

To determine the expression and secretion of IL-1α in MDAPanc-28, ASPC-1, and PANC-1 cells, we performed Northern and Western blot analyses. In MDAPanc-28 and ASPC-1 cells, high levels of IL-1α mRNA were expressed, but its expression was not detectable in PANC-1 cells (Fig. 3A). On Western blot analysis, high levels of IL-1α protein were detected in both cell extracts (50 μg) and conditioned medium from the metastatic human pancreatic cancer cell lines ASPC-1 and MDAPanc-28 but were not detected in those from PANC-1 cells (Fig. 3B). Although IL-1β mRNA was detected in these pancreatic cancer cell lines, a much higher level of IL-1β mRNA was detected in MDAPanc-28 cells than those in ASPC-1 and PANC-1 cells (Fig. 3A). The level of IL-1β protein was very low and barely detectable in ASPC-1 cells and undetectable in PANC-1 cells using 200 μg of protein extracts with the anti-IL-1β antibody, consistent with the expression patterns of IL-1β in these two cell lines detected in Northern blot analysis. However, none of the conditioned medium from these three cells had detectable IL-1β (data not shown). Much lower levels of IL-1β as compared with the level of IL-1α expressed in ASPC-1 and MDAPanc-28 is consistent with the minor reduction of constitutive NF-κB activities in anti-IL-1β neutralizing antibody completely treated conditioned media from these cells (Fig. 2B). Together these results demonstrate that IL-1α is the primary cytokine secreted by metastatic human pancreatic cancer cells and induces constitutive NF-κB activity in these cells.

Neutralizing Antibody of IL-1α Inhibits the Expression of NF-κB Downstream Target Gene—We previously reported that uPA, which plays important roles in tumor invasion, is regulated by constitutive NF-κB activation in pancreatic cancer cells (40). To determine whether the inhibition of IL-1α-induced NF-κB activation decreases the expression of uPA, we treated ASPC-1 and MDAPanc-28 cells with IL-1α neutralizing antibody. Western blot analysis showed that inhibition of the IL-1α signaling cascade in these cells blocked the expression of uPA (Fig. 4). These results further suggest that IL-1α-regulated NF-κB activity plays a key role in controlling the expression of uPA.

Inhibition of NF-κB by 1xBoM Does Not Alter the NF-κB-inducing Activity of IL-1α in the Conditioned Medium—Our data suggest that IL-1α is the primary cytokine secreted by metastatic human pancreatic cancer cell lines. Since our results showed IL-1α autocrine stimulation activated NF-κB activity constitutively in metastatic pancreatic cancer cell lines and the previous reports showed that IL-1α was not only one of the potent NF-κB inducers but also one of the NF-κB-regulated genes, we asked the following question: which one comes first in these cells, IL-1α overexpression or NF-κB constitutive activation? We had previously established ASPC-1/1xBoM and MDAPanc-28/1xBoM cells by pooling ASPC-1 and MDAPanc-28 cells infected with a retrovirus encoding the phosphorylation mutant of IκBα (S32A,S36A) (IκBαM) to specifically inhibit NF-κB activity (18). As shown in Fig. 5A, the NF-κB constitutive activity in these cells was completely inhibited by IκBαM. However, conditioned medium from ASPC-1/IκBαM and MDAPanc-28/IκBαM cells still activated NF-κB and induced the degradation of IκBα in WT-MEF cells in a time-dependent manner (Fig. 5B and C). Importantly anti-IL-1α neutralizing antibody still inhibited IL-1α-induced NF-κB activity, indicating that IL-1α is the primary cytokine in the conditioned media from ASPC-1/IκBαM and MDAPanc-28/IκBαM cells that activates NF-κB in WT-MEF cells (Fig. 5D). These results suggest that inhibition of NF-κB did not inhibit the expression of IL-1α, which is regulated by an NF-κB-independent mechanism.

Inhibition of NF-κB Decreases but Does Not Completely Inhibit Expression of IL-1α—Although IκBαM-mediated inhibition of NF-κB did not inhibit IL-1α expression completely and did not diminish the ability of IL-1α to induce NF-κB activation,
FIG. 7. Down-regulation of EGFR activity reduced AP-1 activity and levels of IL-1α expression. A, AP-1 and NF-κB activities were determined by EMSA in the nuclear extracts isolated from ASPC-1 and MDAPanc-28 cells treated with and without an anti-EGFR antibody (20 ng/ml) for 48 h. Oct-1 DNA activity was determined for the loading control. The levels of IL-1α and β-actin expression were determined by Western blot analysis using cytoplasmic extracts isolated from the same ASPC-1 and MDAPanc-28 cells used in EMSA as described under “Experimental Procedures.” B, NF-κB and Oct-1 activities were analyzed by EMSA in the WT-MEF cells stimulated with the conditioned media from ASPC-1 and MDAPanc-28 cells treated with or without an anti-EGFR antibody (20 ng/ml) for 48 h. C, AP-1 and Oct-1 activities were determined by EMSA in the nuclear extracts isolated from ASPC-1/IκBαM and MDAPanc-28/IκBαM cells treated with and without an anti-EGFR antibody (20 ng/ml). The
NF-κB Activation in Metastatic Pancreatic Cancer Cells

NF-κB expression in pancreatic cancer cells can be regulated by multiple signaling pathways that lead to the stimulation of NF-κB, which in turn induces IL-1α expression in the pancreatic cancer cell line.

To determine whether EGF stimulation induces expression of IL-1α, we stimulated Panc-1, a nonmetastatic pancreatic cancer cell line, with EGF (100 ng/ml) for 18 h and analyzed the expression of IL-1α. As shown in Fig. 7F, expression of IL-1α was induced by EGF stimulation in Panc-1 cells. The conditioned medium from Panc-1 cells with or without EGF stimulation were used to stimulate wild-type MEF cells, and the results showed that NF-κB was activated by the conditioned medium from EGF-stimulated Panc-1 cells, and anti-IL-1α neutralizing antibody only partially inhibited the conditioned levels of IL-1α and β-actin expression were determined by Western blot analysis using cytoplasmic extracts isolated from the same ASPC-1/ΔβcαM and MDAPanc-28/ΔβcαM cells used in EMSA as described under “Experimental Procedures.” D, NF-κB and Oct-1 activities were analyzed by EMSA in the WT-MEF cells stimulated with the conditioned medium from ASPC-1/ΔβcαM and MDAPanc-28/ΔβcαM cells treated with or without an anti-EGFR antibody (20 ng/ml). E, AP-1 reporter gene assays. 1.0 μg of AP-1 reporter gene constructs and the control p-TK Renilla luciferase were cotransfected into Aspc-1 cells with 1.5 μg of expression vectors encoding dominant-negative Elk-1 (ΔElk-1) and ras (rasN17) and kinase-dead AKT and MEKK-1. As shown in Fig. 7E, these results suggest that growth factor-dependent and/or independent activation of mitogen-activated protein kinase, phosphatidylinositol 3-kinase, and other signaling pathways are involved in
medium-induced NF-κB activation (Fig. 7G). The partial inhibition of NF-κB activation by the IL-1α neutralizing antibody may be due to the presence of either residual EGF or additional cytokines in conditioned medium. Taken together these results suggest that the IL-1α expression was induced by EGF in a nonmetastatic pancreatic cancer cell line Panc-1.

Expression of IL-α Is Regulated by AP-1 and NF-κB—To determine whether both AP-1 and NF-κB take part in regulating IL-1α expression, we sought to clone human IL-1α promoter regions. To date, most studies of the IL-1α promoter have been based on a previously reported IL-1α cDNA sequence (National Center for Biotechnology Information accession number X02851) (46). However, our reverse transcription-PCR results suggested that the IL-1α transcription starting site is farther upstream than the previously identified site (data not shown) (46). To verify this finding, we performed primer extension assays. Our results show that the newly identified transcription starting site was actually 458 bp upstream of the previous reported starting site (Fig. 8, A and B) (46). There was one κB-like site at −293 and two AP-1 binding sites at −367 and −22 in this fragment of the 623-bp newly identified IL-1α promoter region (Fig. 8, A and B). A schematic diagram of IL-α promoter and reporter gene constructs with wild-type and mutant AP-1 sequences indicated is shown in Fig. 9A. To determine whether these two AP-1 elements (AP1-1 and AP1-2) in

Fig. 9. IL-1α promoter is regulated by AP-1 activity. A, the fragment of newly identified 623-bp IL-1α promoter region was cloned into pGL2 firefly luciferase reporter gene vector; the two AP-1 sites were mutated as indicated by the bold letter. The arrows indicate the orientation of the AP-1 sites. B, EMSA was performed using MDAPanc-28 nuclear extracts with oligonucleotides encoding the wild-type and mutant AP-1 sites in IL-1 promoter (AP1-1 and AP1-2) as shown in A. The Fos supershift assays with and without competing peptide showed the presence of c-Fos in the sample that contained AP-1 DNA binding activity, and the Oct-1 DNA binding activities were determined as loading controls. C, reporter gene analysis for IL-1α promoter. 1.0 μg of wild-type or mutant IL-1α reporter gene constructs were cotransfected with 1.5 μg of wild-type c-fos, relA(p65), or dominant-negative c-fos into relA−/− cells in a 12-well plate using p-TK Renilla luciferase as control as indicated. The experiments were repeated in triplicate. The activities of both firefly and Renilla luciferase were determined using the dual luciferase reporter assay system (Promega). The luciferase activities were normalized to the Renilla luciferase activity of the internal control. Data represent the means ± S.E. from three different experiments performed in triplicate. Luc, luciferase; D.N., dominant-negative; mut, mutant; pep, peptide.
IL-1 promoter interact with Fos/AP-1, we performed EMSA with wild-type and mutant AP1-1 and AP1-2 probes. The competition and supershift assays showed the presence of c-Fos in the sample that contained AP-1 DNA binding activity (Fig. 9B). These results suggest that IL-1α is likely to be one of the target genes regulated by AP-1 proteins. On the basis of these results, we performed IL-1α promoter luciferase reporter gene assays to determine whether these two AP-1 sites identified in IL-1α promoter are functional. Since pancreatic cancer cells have poor efficiency for transient transfection and to better study the effect of AP-1 and NF-κB on IL-1α transcriptional activity, we selected relA<sup>−/−</sup> MEF cells for the reporter gene analysis. We found that RelA (p65) activated this IL-1α promoter reporter gene construct, whereas dominant-negative c-Fos inhibited IL-1α promoter activity (Fig. 9C). Overexpression of c-Fos induced strong luciferase reporter gene activity in the reporter gene construct with wild-type AP-1 sites but not in the reporter gene constructs with the double mutant AP-1 sites, MAP1-12 (Fig. 9C). The basal IL-1α promoter activity was also inhibited by the presence of the mutant AP-1 sites, mitogen-activated protein kinase (MAP1-1, MAP1-2, and MAP1-12 (Fig. 9C). These results demonstrate that both AP-1 and NF-κB regulate IL-1α expression. Thus, our findings suggest that the constitutive NF-κB activation induced by IL-1α autocrine stimulation in these pancreatic cancer cell lines may be initiated by EGF-dependent or -independent signaling cascade-mediated AP-1 activity.

**DISCUSSION**

Pancreatic adenocarcinoma is the fourth leading cause of adult cancer death in the United States. The 5-year survival rate continues to be 1–3% (47). At the time of diagnosis, most patients with pancreatic cancer have advanced and metastatic disease (48). Studies have suggested that a genetic profile for pancreatic cancer is emerging based on the most frequently detected mutations in this disease (49). K-ras mutation is an early event in pancreatic carcinogenesis that has been detected in 80–95% of pancreatic cancers, overexpression of EGR1 occurs in ~90% of human pancreatic tumors, and inactivation of Smad4, Ink4a/Arf, and p53 tumor suppressor genes has been identified in ~50–75% of pancreatic cancers (49). However, the role of specific genetic alterations that initiate tumorigenesis and mediate its cardinal clinical features of locally aggressive growth, metastasis, and chemotherapy resistance remains to be elucidated.

Recently we demonstrated that RelA, the p65 subunit of NF-κB transcription factors, is constitutively activated in most human pancreatic cancer tissues and cell lines (17), and this activation has been shown to play key roles in pancreatic tumor progression and liver metastasis of pancreatic adenocarcinoma (19, 20). Therefore, understanding the mechanisms by which NF-κB is constitutively activated in pancreatic cancer cells will elucidate the signaling cascades that directly or indirectly regulate NF-κB activation.

In this study, we found that the autocrine mechanism is responsible for the constitutive activation of NF-κB in metastatic human pancreatic cancer cell lines but not in nonmetastatic ones. Subsequent studies showed that IL-1α is the primary NF-κB-activating cytokine secreted by these cell lines. IL-1α has been shown to play important roles in proinflammatory responses, cancer cell growth, and metastasis (15, 50–52), and it has been suggested that IL-1α participates in cancer cell invasion and metastasis by regulating the expression of some adhesion molecules. For instance, IL-1α could increase the expression of MMP-9, E-selectin, integrin-1, and IL-8 and inhibit plasminogen activator inhibitor-1 (34, 50–53). A recent report suggested that IL-1α enhances the adhesion of metastatic human pancreatic cancer cells to extracellular matrix proteins but not nonmetastatic ones (53). Our findings showed that IL-1α is the primary cytokine that causes constitutive NF-κB activation in metastatic human pancreatic cancer cells and that blocking IL-1α in these cells inhibited the expression of uPA (Fig. 4). Based on these data and our previous findings that constitutively activated NF-κB plays key roles in pancreatic tumorigenesis and metastasis and that overexpression of uPA in pancreatic adenocarcinoma is regulated by constitutively activated RelA (19, 20, 40), one possible mechanism by which IL-1α participates in pancreatic tumorigenesis and cancer metastasis is the IL-1α-regulated expression of a number of key determinants for tumorigenic and metastatic phenotype through NF-κB.

IL-1α is a key mediator of the inflammatory response (54). It is not only a potent inducer of NF-κB but also a downstream target gene of NF-κB (25, 30). This led us to an interesting question. Which of the molecular alteration, overexpression of IL-1α, or constitutive activation of NF-κB proceeds during the development of pancreatic cancer cells? In this study, we demonstrated that expression and secretion of IL-1α were decreased but not completely blocked by the IxBα/M-mediated inhibition of NF-κB and that IL-1α present in the conditioned medium isolated from both ASPC-1/IxBαM and MDAPanc-28/IxBαM cells is still capable of activating NF-κB in WT-MEF cells, similar to that isolated from ASPC-1 and MDAPanc-28 cells. These findings suggest that expression of IL-1α is primarily regulated by NF-κB-independent mechanisms in human pancreatic cancer cells. The constitutive NF-κB activity triggered by IL-1α autocrine stimulation may enhance the IL-1α expression. Thus, this may initiate a possible formation of a positive feedback loop and connect inflammatory responses and cancer development.

**Fig. 10. A working model for constitutive NF-κB activation in metastatic pancreatic cancer cells.** In metastatic pancreatic cancer cells, growth factors and/or other upstream signal cascades trigger AP-1 activation, which in turn induces the expression of its downstream target genes such as IL-1α. The IL-1α autocrine stimulation activates NF-κB, which further elevates the expression of its downstream target gene, IL-1α, thus resulting in a positive feedback loop and causing NF-κB to be constitutively activated. The constitutively activated NF-κB regulates its downstream target genes, promoting pancreatic tumor progression and metastasis.
NF-κB Activation in Metastatic Pancreatic Cancer Cells

Previous studies have shown that the expression of IL-1α is regulated by NF-κB and AP-1 transcription factors (25, 42). In our newly identified IL-1α promoter region, there are multiple NF-κB and AP-1 sites, and our reporter gene analysis confirmed that IL-1α is regulated by both NF-κB and AP-1. IL-1α has also been found to induce AP-1 activity (32). These findings suggest that IL-1α autocrine stimulation may be initiated through AP-1 activation.

The recently proposed progression model of pancreatic adenocarcinoma suggests a profile of genetic alterations that includes overexpression of EGFR and mutations of the K-ras gene (49). EGFR and K-ras may induce AP-1 activation and IL-α overexpression in pancreatic cancers. Studies have shown that EGFR induced AP-1 activity, and both EGFR tyrosine kinase activity and autophosphorylation at Tyr-1173 play a critical role in EGFR-induced AP-1 activation (55). Several reports have suggested that the Ras signaling pathway induces AP-1 activation and regulates IL-1α expression (56–58). Consequently, autocrine production of IL-1α induces the transcriptional activation of both NF-κB and AP-1. These findings imply that genetic alterations in the development of pancreatic adenocarcinoma possibly initiate the overexpression of IL-1α, which, in turn, induces a large number of the downstream target genes that encode the key determinants of tumorigenic and metastatic phenotypes through activation of NF-κB and AP-1 transcription factors. The working model for constitutive NF-κB activation in metastatic pancreatic cancer cells is illustrated in Fig. 10.

In summary, we identified the proinflammatory cytokine IL-1α as the primary cytokine that induces constitutive NF-κB activation in metastatic human pancreatic cancer cell lines. The constitutive NF-κB activity in turn enhances the expression of IL-1α, suggesting that this autocrine mechanism plays an important role in the development of the tumorigenic and metastatic phenotype, making a positive feedback loop possible. Autocrine production of IL-1α may be primarily induced by AP-1 activity, and AP-1 is a potential target for IL-1α, suggesting that an additional positive feedback loop may be possible between IL-1α expression and AP-1 activity. Many studies have shown that both NF-κB and AP-1 are activated in response to growth factors and inflammatory stimuli, and constitutive activation of NF-κB and AP-1 has been shown to be critical in the development of cancer. Therefore, our results also suggest that NF-κB and AP-1 might be the missing mechanistic links between inflammation and cancer.

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