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Radiation to Stromal Fibroblasts Increases Invasiveness of Pancreatic Cancer Cells through Tumor-Stromal Interactions[1]

Kenoki Ohuchida,[1] Kazuhiro Mizumoto,[1] Mitsuhiko Murakami,[1] Li-Wu Qian,[1] Norihiro Sato,[1] Eishi Nagai,[1] Kunio Matsumoto,[2] Toshikazu Nakamura,[2] and Masao Tanaka[1]

[1]Department of Surgery and Oncology, Graduate School of Medical Sciences, Kyushu University, Fukuoka, Japan; and [2]Division of Molecular Regenerative Medicine, Course of Advanced Medicine, Osaka University Graduate School of Medical Science, Suita, Osaka, Japan

ABSTRACT

Radiotherapy represents a major treatment option for patients with pancreatic cancer, but recent evidence suggests that radiation can promote invasion and metastasis of cancer cells. Interactions between cancer cells and surrounding stromal cells may play an important role in aggressive tumor progression. In the present study, we investigated the invasive phenotype of pancreatic cancer cells in response to coculture with irradiated fibroblasts. Using *in vitro* invasion assay, we demonstrated that coculture with nonirradiated fibroblasts significantly increased the invasive ability of pancreatic cancer cells and, surprisingly, the increased invasiveness was further accelerated when they were cocultured with irradiated fibroblasts. The hepatocyte growth factor (HGF) secretion from fibroblasts remained unchanged after irradiation, whereas exposure of pancreatic cancer cells to supernatant from irradiated fibroblasts resulted in increased phosphorylation of c-Met (HGF receptor) and mitogen-activated protein kinase activity, possibly or partially via increased expression of c-Met. We also demonstrated that scattering of pancreatic cancer cells was accelerated by the supernatant from irradiated fibroblasts. The enhanced invasiveness of pancreatic cancer cells induced by coculture with irradiated fibroblasts was completely blocked by NK4, a specific antagonist of HGF. These data suggest that invasive potential of certain pancreatic cancer cells is enhanced by soluble mediator(s) released from irradiated fibroblasts possibly through up-regulation of c-Met expression/phosphorylation and mitogen-activated protein kinase activity in pancreatic cancer cells. Our present findings further support the potential use of NK4 during radiotherapy for patients with pancreatic cancer.

INTRODUCTION

Pancreatic cancer is a leading cause of cancer-related deaths in the industrialized countries (1, 2). A vast majority of patients with pancreatic cancer have poor outcomes because of difficulties in early diagnosis and limited efficiency of conventional therapeutics such as surgical resection, chemotherapy, and radiotherapy (3).

Radiotherapy is one of the major adjuvant treatments for many malignant tumors and has been frequently applied for patients with pancreatic cancer. The rationale for radiotherapy is based on the findings that radiation can inhibit cell proliferation or induce apoptotic cell death *in vitro* and inhibit tumor growth *in vivo* (4). Recently, however, several lines of evidence have shown that irradiation promotes malignant behaviors of cancer cells *in vitro* and *in vivo* by activating several pathways involved in tumor invasion and metastasis (5–9). We have previously reported that irradiation enhances invasive potential of pancreatic cancer cells via an increased expression of matrix metalloproteinase (MMP)-2 or c-Met/hepatocyte growth factor (HGF) receptor in cancer cells (10, 11).

Interactions between cancer cells and surrounding stromal fibroblasts have been suggested to play a critical role in tumor invasion and metastasis (12, 13). For example, invasive growth of certain cancer cells is drastically enhanced by tumor-stromal interactions involving the HGF/c-Met (HGF receptor) pathway (14–16). HGF, which was originally identified as a potent mitogen for hepatocytes (17, 18), is primarily secreted from stromal cells, and the secreted HGF can promote motility and invasion of various cancer cells that express c-Met in a paracrine manner (19–21). Binding of HGF to c-Met leads to receptor phosphorylation and activation of Ras/mitogen-activated protein kinase (MAPK) signaling pathway, thereby enhancing malignant behaviors of cancer cells (22, 23).

Infiltrating ductal adenocarcinoma of the pancreas is often characterized by an abundant desmoplastic stroma. Nevertheless, the effects of radiation to the stromal components have not been studied. We hypothesized that irradiation to stromal cells as well as to cancer cells themselves could affect the invasive phenotype of pancreatic cancer through modifying tumor-stromal interactions. To test this hypothesis, we investigated the invasive behavior of pancreatic cancer cells in response to coculture with irradiated fibroblasts. We also determined the concentration of a panel of growth factors including HGF in culture supernatant from fibroblasts after irradiation and expression/phosphorylation of c-Met and MAPK activity in pancreatic cancer cells exposed to the supernatant from irradiated fibroblasts.

MATERIALS AND METHODS

Cells and Reagents. Three human pancreatic cancer cell lines [Suit-2, generously provided by Dr. H. Iguchi (National Kyushu Cancer Center, Fukuoka, Japan) and Capan-1 and SW1990, purchased from American Type Culture Collection (Rockville, MD)], one human fibroblast cell line [MRC5, from Riken (Tokyo, Japan)], and primary pancreatic fibroblasts derived from a patient with invasive pancreatic adenocarcinoma were used in this study. Cells were maintained in DMEM (Sigma Chemical Co., St. Louis, MO) supplemented with 10% fetal bovine serum (FBS), streptomycin (100 μg/ml), and penicillin (100 units/ml) at 37°C with humidified 90% air and 10% CO_2. Human recombinant NK4 was purified from the conditioned medium of Chinese hamster ovary cells transfected with expression vector for human NK4 cDNA as described previously (24). Polyclonal anti-HGF antibody was prepared as described previously (25). Anti-fibroblast growth factor (FGF) basic antibody was purchased from R & D Systems (Minneapolis, MN).

Radiation. Cells were irradiated with a dose of 5 or 10 Gy at room temperature using a ^{137}Cs source (Gamma Cell 40; Atomic Energy of Canada, Ltd., Ontario, Canada) with a delivering rate of 1.0 Gy/min. MRC5 fibroblasts were seeded at a density of 1.5×10^6 cells in 90-mm dishes and irradiated at 5 or 10 Gy. After cultivation for 24 h, the supernatant of these fibroblasts were harvested, centrifuged to remove debris, and then used to stimulate pancreatic cancer cells.

Invasion Assay. Invasion of pancreatic cancer cells was measured by the number of cells invading through Matrigel-coated transwell inserts (Becton Dickinson, Franklin Lakes, NJ) as reported previously (26). In brief, transwell inserts with 8-μm pores were coated with Matrigel (20 μg/well; Becton Dickinson). Fibroblasts were initially seeded on a 24-well plate at a density of 2.5×10^4/cm^2 and cultured in DMEM supplemented with 10% FBS for 24 h.

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Requests for reprints: Kazuhiro Mizumoto, Department of Surgery and Oncology, Graduate School of Medical Sciences, Kyushu University, 3-1-1 Maidashi, Higashi-ku, Fukuoka 812-8582, Japan. Phone: 81-92-642-5440; Fax: 81-92-642-5458; E-mail: mizumoto@med.kyushu-u.ac.jp.

These fibroblasts were then irradiated at 5 or 10 Gy, and the medium was immediately replaced with 750 μl of fresh medium. Cancer cells were seeded at a density of $1 \times 10^5/cm^2$ in the upper chambers with 250 μl of DMEM supplemented with 10% FBS with or without human recombinant NK4, anti-HGF antibody, or anti-FGF basic antibody at an indicated final concentration. After 24 h of incubation, cells that had invaded to lower surface of the Matrigel-coated membrane were fixed with 70% ethanol, stained with H&E, and counted in five randomly selected fields under a light microscope.

Immunoblot Analysis for c-Met and Its Phosphorylated Type (p-Met). Pancreatic cancer cells were cultured with supernatant of irradiated or nonirradiated MRC5 fibroblasts for the indicated hours. For c-Met detection, cells cultured in 90-mm dishes were directly lysed in a lysis buffer (900 μl) composed of 50 mM NaCl, 10 mM Tris-HCl (pH 7.4), 5 mM EDTA, 30 mM sodium PP_i, 50 mM NaF, 0.5 μg/ml leupeptin, 1 mM phenylmethyl sulfonylfluoride, and 1% Triton X-100. The whole cell lysates were fractionated by 8% SDS-PAGE and transferred to a polyvinylidene difluoride membrane (Millipore, Bedford, MA). The membrane was incubated with 1:500–1,000 dilutions of antihuman c-Met polyclonal IgG specific for the COOH terminus of c-Met p140 (C-12; Santa Cruz Biotechnology, Santa Cruz, CA) and then probed with antirabbit IgG conjugated with horseradish peroxidase (Santa Cruz Biotechnology).

For c-Met tyrosine phosphorylation, the cells were washed with PBS and lysed in an ice-cold lysis buffer [10 mM Tris-HCl (pH 7.4), 150 mM NaCl, 2 mM EGTA, 2 mM DTT, 1 mM orthovanadate, 1 mM phenylmethyl sulfonylfluoride, 1 μg/ml leupeptin, and 10 μg/ml aprotinin (pH 7.4)]. The cell lysates were separated by 8% SDS-PAGE under reducing conditions. The phosphorylated Met was probed by a polyclonal IgG recognizable for phosphorylation site in amino acids 1231–1240 of human c-Met (Y1234 and Y1235; Upstate Biotechnology, Lake Placid, NY). Immunoblots were detected by enhanced chemiluminescence (Amersham Biosciences, Little Chalfont, UK) and developed by Hyperfilm (Amersham Biosciences). The relative density of each band was determined with Image Gauge software (version 3.01; Fuji Photo Film Co., Ltd. and Kohshin Graphic Systems, Inc., Tokyo, Japan).

p42/p44 MAP Kinase Enzyme Assay. MAPK activity was assayed using the peptide phosphorylation assay kit (code RPN 84; Amersham Biosciences). The assay used as substrate a peptide containing a single phosphorylation site corresponding to the MAPK phosphorylation consensus sequence, Pro-Leu-Thr-Pro, found in epidermal growth factor receptor at Thr^{669}. Cells were grown subconfluent in 6-well plates and then cultured with the supernatant of irradiated or nonirradiated fibroblasts for the indicated hours. Cells were washed in ice-cold PBS and lysed in 0.3 ml of 10 mM Tris-HCl (pH 7.4), 150 mM NaCl, 2 mM EGTA, 2 mM DTT, 1 mM orthovanadate, 1 mM phenylmethyl sulfonylfluoride, 1 μg/ml leupeptin, and 10 μg/ml aprotinin. After centrifugation of the cell lysate at 16,000 rpm for 30 min, 15-μl aliquots were used for the assay. MAP kinase activity was determined as the amount of ^{32}P incorporation, which was quantitatively read by a liquid scintillation system (LSC-3500; Aloka, Tokyo, Japan). At least four measurements were done for each sample.

Scattering Assay. Pancreatic cancer cells were plated at a density of 250 cells/well in DMEM containing 10% FBS into 6-well plates. After cultivation for 5 days, cells were stimulated by the supernatant of irradiated or nonirradiated MRC5 cells. Scattering of cells was evaluated under a phase-contrast microscope (DIAHOT; Nikon, Inc., Tokyo, Japan) at 24 h after stimulation.

Laser-Scanning Confocal Microscopy for Immunofluorescence Staining of c-Met. Pancreatic cancer cells were plated on 24-well chamber slides (Lab-Ted Nunc, Inc., Naperville, IL) and were stimulated by the supernatant from irradiated or nonirradiated MRC5 fibroblasts. The cancer cells were then incubated for 8 h, fixed with cold methanol for 5 min, blocked with a blocking solution (10% normal goat serum, 3% BSA, and 0.5% gelatin in PBS) and incubated with the c-Met antibody (C-12; Santa Cruz Biotechnology) for 30 min at 37°C. The cells were then incubated for 30 min with Alexa 488-conjugated antirabbit IgG (Molecular Probes, Eugene, OR). Nuclear DNA was counterstained with propidium iodide. Slides were mounted with Gel/Mount (Biomeda Corporation, Foster City, CA) and visualized under a laser-scanning confocal fluorescent microscope (LSM-GB200 System; Olympus Optical Corp., Tokyo, Japan).

ELISA. ELISA was performed using a human HGF enzyme immunoassay kit (Special Immune Institute, Tokyo, Japan) for HGF, Biotrak human ELISA (Amersham Biosciences) for vascular endothelial growth factor, and a Quantikine kit (R & D Systems) for epidermal growth factor, FGF, and transforming growth factor β1 according to the manufacturer's instructions. MRC5 cells and primary fibroblasts were seeded at a density of 2×10^5/well on 6-well plates in 2 ml of DMEM containing 10% FBS and cultured for 24 h. Culture supernatants were harvested from irradiated or nonirradiated fibroblasts after 24 h of incubation and subjected to the measurements for each growth factor.

Reverse Transcription-PCR for HGF and c-met mRNA Expression. Total RNA was extracted from cells using an Isogen-based method (Nippongene, Inc., Toyama, Japan). The reverse-transcription from RNA to cDNA and PCR amplification were performed using an RNA PCR kit (TaKaRa Biomedicals, Kyoto, Japan), according to the manufacturer's instructions. Primers for amplification of the c-met gene and HGF gene were designed as described previously (27). Thirty-five cycles were performed, each consisting of 94°C for 1 min, 60°C for 1 min, and 72°C for 1 min. An aliquot of the reaction was then analyzed by 2% agarose gel electrophoresis. The sizes of the predicted products should be 523 bp for HGF and 222 bp for c-Met.

***Ex vivo* Model.** MRC5 cells and pancreatic fibroblasts were irradiated at 10 or 5 Gy, respectively, just before implantation. Suit-2 cells (1×10^6) and 1×10^6 irradiated or nonirradiated fibroblasts were mixed well and implanted into the pancreas of 6-week-old female nude mice (BALB/c nu/nu; Kyoudo Co., Ltd., Saga, Japan). Three mice were used in each group. Seven days after implantation, tumors were resected with the surrounding tissue. Tissues were fixed in 10% neutral formalin for 24 h and embedded in paraffin according to standard histological procedures. These tissue sections were washed in PBS and stained with H&E for histopathological examinations. To semiquantitate the invasiveness of the implanted pancreatic cancer, we defined the invasion score based on histological observations as previously reported (24): score 0, invasion was undetectable, and the tumor was surrounded by a capsule; score 1, invasion was undetectable, but the tumor was not surrounded by a capsule; score 2, invasion was partial; score 3, invasion was extensive, and normal pancreatic and tumor regions could not be distinguished.

Immunohistochemical Studies. Fixed tumor tissue sections were embedded in paraffin. These tissue sections were quenched with 3% hydrogen peroxide in PBS for 5 min, washed in PBS, and treated with 0.1% trypsin at room temperature for 5 min. The sections were exposed for 30 min to 10% normal goat serum and incubated with rabbit anti-c-Met phosphospecific antibodies (diluted 1:50; BIOSOURCE) overnight at 4°C. Next, the sections were incubated with biotinylated peroxidase-conjugated goat antirabbit IgG antibodies (DAKO Co., Carpinteria, CA) for 60 min. The reaction was observed by incubating the sections with substrate solution containing diaminobenzidine and hydrogen peroxide. The sections were then washed in PBS and counterstained with hematoxylin.

Statistical Analysis. Values were expressed as the mean ± SD. Comparisons between three or more groups were done by one-way ANOVA and by the Student's *t* test for comparison between two groups. The level of statistical significance was set at $P < 0.01$.

RESULTS

Irradiated Fibroblasts Promote the Invasiveness of Nonirradiated Pancreatic Cancer Cells. Before investigating the effect of irradiation on invasiveness of cancer cells, we examined the effect of 5-Gy or 10-Gy irradiation on proliferation of cancer cells (Suit-2 cells) and fibroblasts (MRC5 cells and pancreatic fibroblasts) using propidium iodide assay as described in our previous report (10). The cells were not cytolytic 24 h after radiation and cytostatic in a dose-dependent manner. The proliferation of cancer cells was completely inhibited at the dose of 10 Gy, whereas that of fibroblasts was only partially inhibited at the same dose.

Using *in vitro* invasion assay, we examined the invasiveness of pancreatic cancer cells cocultured with MRC5 fibroblasts that were nonirradiated, irradiated at 5 Gy, or irradiated at 10 Gy. Only a small number of Suit-2 pancreatic cancer cells invaded through Matrigel when they were cultured alone, whereas coculture with MRC5 fibroblasts increased the invasive ability of Suit-2 cells, which is consistent with our previous data (28). Surprisingly, coculture with MRC5 cells after irradiation further enhanced the invasiveness of Suit-2 cells in a

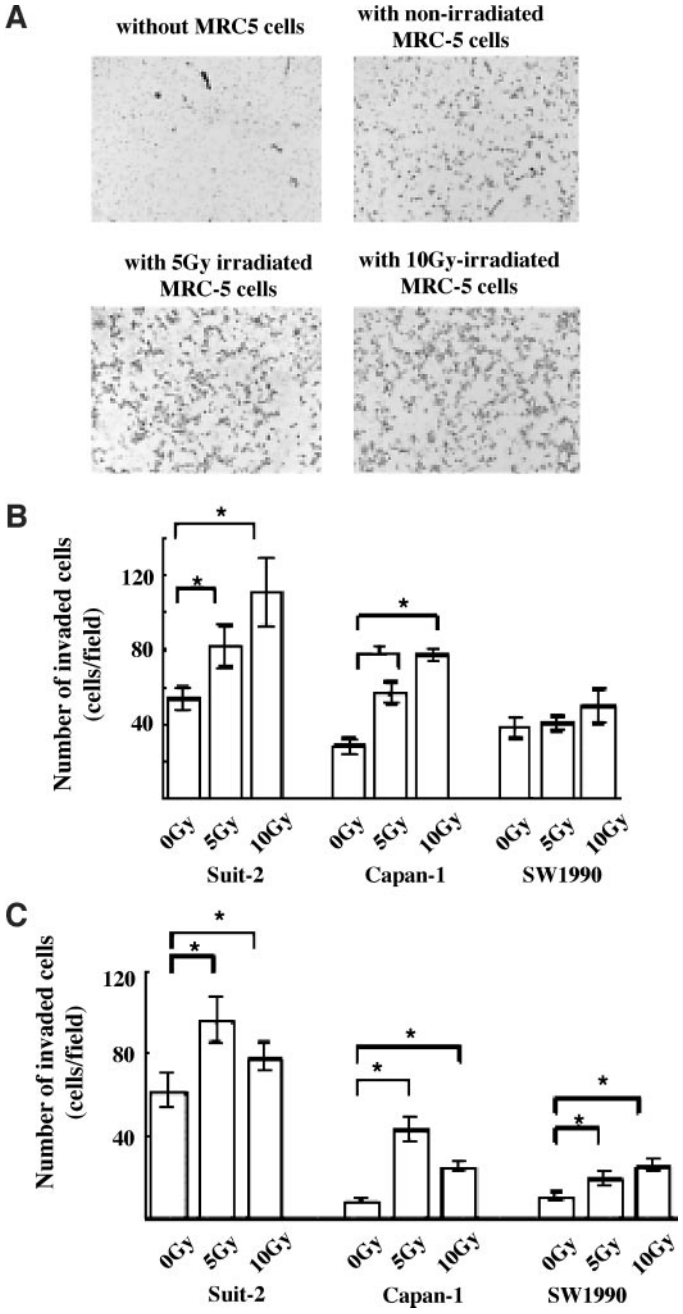

Fig. 1. Effects of coculture with irradiated fibroblasts on the invasiveness of human pancreatic cancer cells. *A,* photomicrographs of *in vitro* invasion assay in Suit-2 cells cocultured with nonirradiated or irradiated MRC5 fibroblasts (at 5 or 10 Gy). H&E stain, ×40. *B,* invasive potential (as determined by the number of invaded cells) of three pancreatic cancer cell lines (*Suit-2, Capan-1,* and *SW1990*) cocultured with irradiated MRC5 fibroblasts. *C,* invasive potential of three pancreatic cancer cell lines cocultured with irradiated primary pancreatic fibroblasts. Each value represents the mean ± SD of triplicate measurements. *, $P < 0.01$

dose-dependent manner (Fig. 1*A*). The number of invading Suit-2 cells cocultured with MRC5 cells irradiated at 10 Gy was 2.1-fold greater than the number of invading Suit-2 cells cocultured with nonirradiated MRC5 cells (Fig. 1*B*). Similar results were obtained in Capan-1 cells but not in SW1990 cells; irradiation to MRC5 fibroblasts further accelerated the invasion of Capan-1 in a dose-dependent manner in a coculture system but not of SW1990 (Fig. 1*B*).

To rule out the possibility that the enhanced invasiveness by irradiation to fibroblasts was a specific event for MRC5 cells, we next tested the invasiveness of pancreatic cancer cell lines cocultured with primary pancreatic fibroblasts derived from resected specimen from a patient with invasive pancreatic adenocarcinoma. Coculture with irradiated primary pancreatic fibroblasts (at 5 and 10 Gy) showed significantly larger number of invading cells compared with coculture with nonirradiated counterparts in all three pancreatic cancer cell lines tested (Suit-2, Capan-1, and SW1990; Fig. 1*C*).

Irradiated Fibroblasts Further Promote the Invasiveness of Irradiated Pancreatic Cancer Cells. We previously documented that the invasiveness of human pancreatic cancer cells including AsPC1 and Panc1 was enhanced after irradiation (10). We therefore investigated the invasiveness of irradiated or nonirradiated Suit-2 cells in coculture with irradiated or nonirradiated fibroblasts. Suit-2 cells irradiated at 5 Gy showed a slight increase in the number of invading cells when they were cocultured with nonirradiated MRC5 cells (Fig. 2). In contrast, coculture of nonirradiated Suit-2 cells with MRC5 cells that had received the same dose (5 Gy) of irradiation increased the number of invading Suit-2 cells. Coculture of irradiated Suit-2 and irradiated MRC5 cells showed the largest number of invading cells, but the increase in the number of invading cells was modest compared with coculture of nonirradiated Suit-2 and irradiated MRC5 (Fig. 2*B*). These results suggest that radiation to fibroblasts rather than to pan-

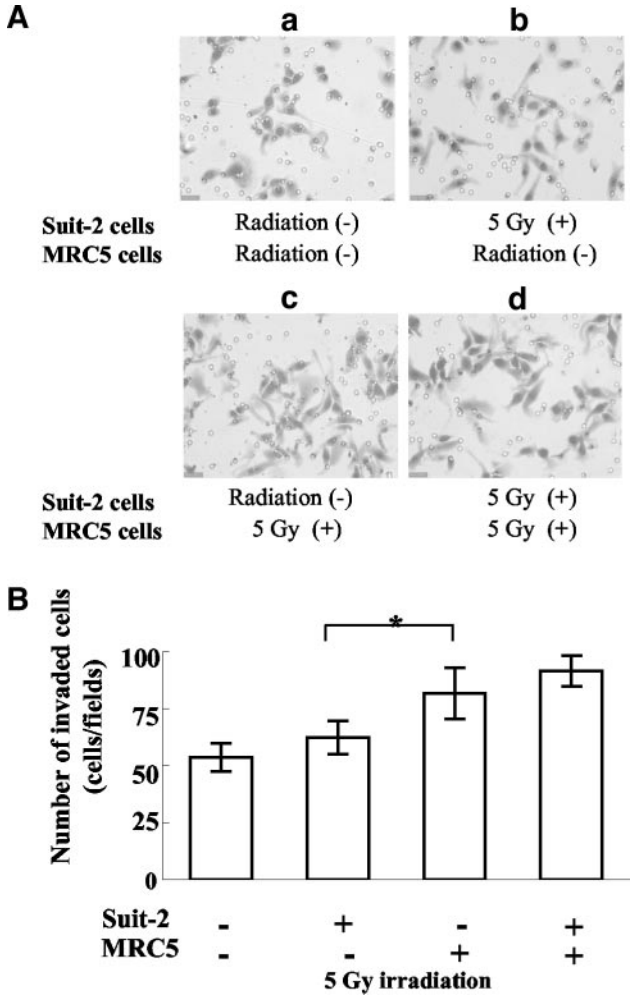

Fig. 2. Effects of coculture of nonirradiated or irradiated pancreatic cancer cells and nonirradiated or irradiated fibroblasts on the invasion of pancreatic cancer cells. *A,* photomicrographs of *in vitro* invasion assay in nonirradiated or irradiated (5 Gy) Suit-2 cells cocultured with either nonirradiated or irradiated (5 Gy) MRC5 fibroblasts. H&E stain, ×200. *B,* invasive potential (as determined by the number of invaded cells) of nonirradiated or irradiated (5 Gy) Suit-2 cells cocultured with either nonirradiated or irradiated (5 Gy) MRC5 fibroblasts. Each value represents the mean ± SD of triplicate measurements. *, $P < 0.01$

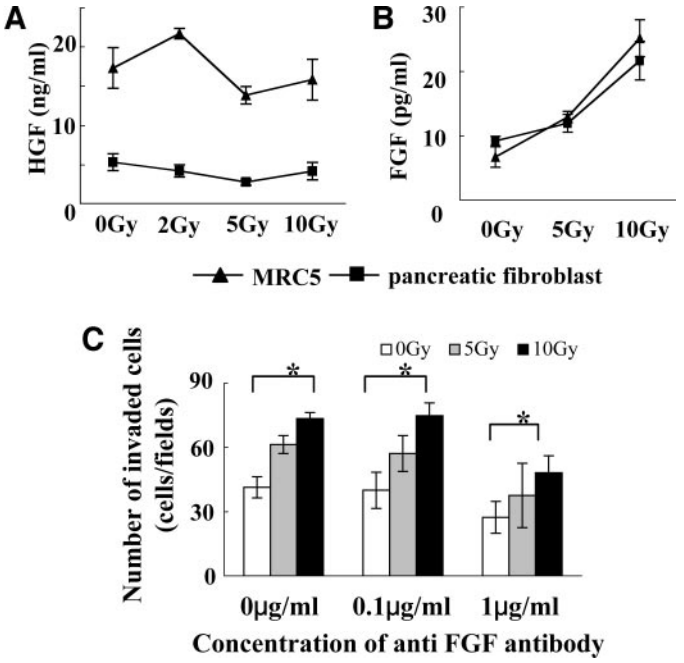

Fig. 3. The concentration of *HGF* (*A*) and basic-*FGF* (*B*) in the supernatant of fibroblasts (MRC5 or primary pancreatic fibroblasts) after irradiation. Fibroblasts were irradiated at 2, 5, or 10 Gy and cultured for 24 h in a fresh serum-containing medium, and the supernatant was subjected to ELISA to measure the concentration of these growth factors. *C,* the number of invaded Suit-2 cells cocultured with MRC5 cells in the absence or presence of anti-FGF basic antibody at a concentration of 0.1 or 1 μg/ml. Each value represents the mean ± SD of triplicate measurements. *, $P < 0.01$

creatic cancer cells themselves strongly enhances the invasive behaviors of pancreatic cancer cells in a coculture system.

Changes in the Concentration of Growth Factors and the Expression/Activity of Major Proteolytic Systems in the Supernatant from Irradiated Fibroblasts. To elucidate the mechanism by which coculture with irradiated fibroblasts enhances the invasiveness of pancreatic cancer cells, we measured the concentration of a panel of growth factors (HGF, basic-FGF, transforming growth factor β1, vascular endothelial growth factor, and epidermal growth factor) in the supernatant from fibroblasts after irradiation. The concentration of HGF remained unchanged after irradiation in both MRC5 cells and primary pancreatic fibroblasts (Fig. 3*A*). The concentration of basic FGF in the supernatant of irradiated MRC5 cells and primary pancreatic fibroblasts increased significantly after irradiation (Fig. 3*B*). To confirm whether the modestly increased basic FGF expression is a responsible factor to enhance the invasion potential of cancer cells with a coculture after radiation, the anti-FGF basic antibody was used for invasion assay. As shown in Fig. 3*C,* the anti-FGF basic antibody showed the modest inhibitory effect on the invasion potential independently of irradiation, but the radiation-enhanced invasion potential of Suit-2 cells was still maintained in the presence of 1 μg/ml anti basic FGF antibody, which is sufficient to inhibit bioactivity of 500 pg/ml basic FGF completely. These data suggest that the increase of basic FGF is not critical to induce radiation-enhanced invasiveness in a coculture system, although the presence of FGF is involved in invasion potential.

The concentrations of other growth factors (transforming growth factor β1, vascular endothelial growth factor, and epidermal growth factor) were relatively low in nonirradiated fibroblast cultures (29–31) and did not show any changes after irradiation (data not shown), suggesting that these growth factors may not be involved in the increased invasiveness of cocultured pancreatic cancer cells. We also examined the supernatant from irradiated fibroblasts for the expression of two major proteolytic systems including matrix MMPs and urokinase-type plasminogen activator. We used gelatin zymography to determine the activity and the expression of MMP-2 and MMP-9 and immunoblot analysis to examine the expression of urokinase-type plasminogen activator. The expressions of MMP-2 and MMP-9 were detectable in the fibroblast supernatant, but their expression levels and activities were not changed 8, 16, and 24 h after irradiation (data not shown). The expression of urokinase-type plasminogen activator was not detected in both MRC5 cells and pancreatic fibroblasts before and after irradiation (data not shown).

Change in MAPK Activity in Suit-2 Cells Exposed to the Supernatant of Irradiated Fibroblasts. MAPK is known to be activated by various growth factors including HGF and basic FGF (32), and activation of this signaling pathway has been shown to play a crucial role in the acquisition of invasive and metastatic phenotype of cancer cells (22, 23). We therefore measured the MAPK activity in Suit-2 cells after exposure to the supernatant from either irradiated or nonirradiated MRC5 fibroblasts. The MAPK activity kinetics in Suit-2 after exposure to the supernatant from nonirradiated MRC5 cells showed an increased activity as a single peak at ~30 min after the beginning of exposure (Fig. 4). In contrast, the MAPK activity kinetics in Suit-2 cells exposed to the supernatant of irradiated MRC5 cells showed a biphasic pattern of increase identified as two distinct peaks at ~30 min and ~8 h after exposure (Fig. 4). The first peaks were always observed in all experiments, but the degree of these peaks was not reproducible. However, the second peaks observed only in irradiated experiments were always reproducible. Notably, the MAPK activity in Suit-2 exposed to the supernatant from irradiated fibroblasts reached to ~4-fold at the second peak compared with the activity observed in Suit-2 cells exposed to the supernatant from nonirradiated fibroblasts. Thus, the increase in the MAPK activity at the late phase may be associated with the increased invasion of Suit-2 cells cocultured with irradiated fibroblasts.

Changes in c-Met and p-Met Expression in Suit-2 Cells Exposed to the Supernatant of Irradiated MRC5 Cells. Because interactions between growth factors and their receptors (such as the interaction of HGF and its receptor c-Met) can activate the Ras-Raf-mitogen-activated protein/extracellular signal-regulated kinase kinase-MAPK signal transduction pathway (29, 33, 34), we examined the expression of c-Met and the phosphorylated form of c-Met (p-Met) in Suit-2 cells after exposure to the supernatant from irradiated or nonirradiated fibroblasts. The expression of c-Met in Suit-2 cells increased after exposure to the supernatant from irradiated fibroblasts in a time-dependent manner and reached to approximately 2-fold at 8 h after exposure (Fig. 5). In contrast, the c-Met expression in Suit-2

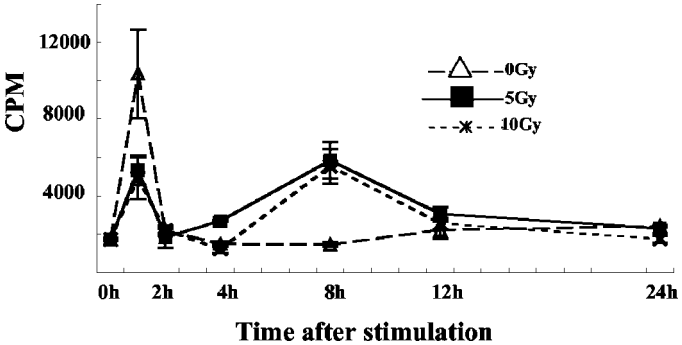

Fig. 4. p44/p42 MAPK(extracellular signal-regulated kinase 1/2) enzyme activity in Suit-2 pancreatic cancer cells exposed to the supernatant from irradiated or nonirradiated MRC5 fibroblasts. A biphasic increase in MAPK activity is observed in Suit-2 cells after exposure to the supernatant from irradiated MRC5 cells, in contrast to the activity of Suit-2 cells exposed to the supernatant from nonirradiated fibroblasts showing a single peak. Suit-2 cells were seeded and cultured for 24 h.

cells exposed to the supernatant from nonirradiated fibroblasts showed a minimal increase at 1 h after stimulation. The expression of c-Met and especially its phosphorylated form (p-Met) was markedly augmented in Suit-2 cells after exposure to the supernatant from irradiated fibroblasts, and the relative intensity of p-Met expression induced by irradiated MRC5 cells increased in a dose-dependent manner to 2.9-fold (at 5 Gy) and 3.6-fold (at 10 Gy) as compared with that induced by nonirradiated MRC5 cells (Fig. 6*A*). The relative intensity of p-Met expression induced by irradiated pancreatic fibroblasts increased to 4.2-fold (at 5 Gy) and 2.4-fold (at 10 Gy) as compared with that induced by nonirradiated pancreatic fibroblasts (Fig. 6*B*). These changes of p-Met were well correlated with the change in the invasion potential observed in invasion assay using both of MRC5 cells and pancreatic fibroblasts (Fig. 1, *B* and *C*). These data strongly support the involvement of phospho-Met in the promoting effect of coculture with irradiated fibroblasts on invasion of cancer cells.

To confirm the increased expression of c-Met, we used indirect fluorescent microscopy to examine the *in situ* expression of c-Met in Suit-2 cells after exposure to the supernatant of irradiated or nonirradiated MRC5 cells. Compared with Suit-2 cells exposed to the supernatant from nonirradiated fibroblasts, the c-Met fluorescence intensity was increased specifically in the cell membrane of Suit-2 at 8 h after exposure to the supernatant of irradiated MRC5 cells (Fig. 7).

The Supernatant from Irradiated MRC5 Cells Promotes Scattering of Suit-2 Cells. We then examined the effect of the supernatant derived from irradiated fibroblasts on cell scattering, which is a well-characterized behavior activated by HGF/c-Met system (35). Exposure to the supernatant from irradiated fibroblasts accelerated the scattering of Suit-2 cells in a dose-dependent manner (Fig. 8).

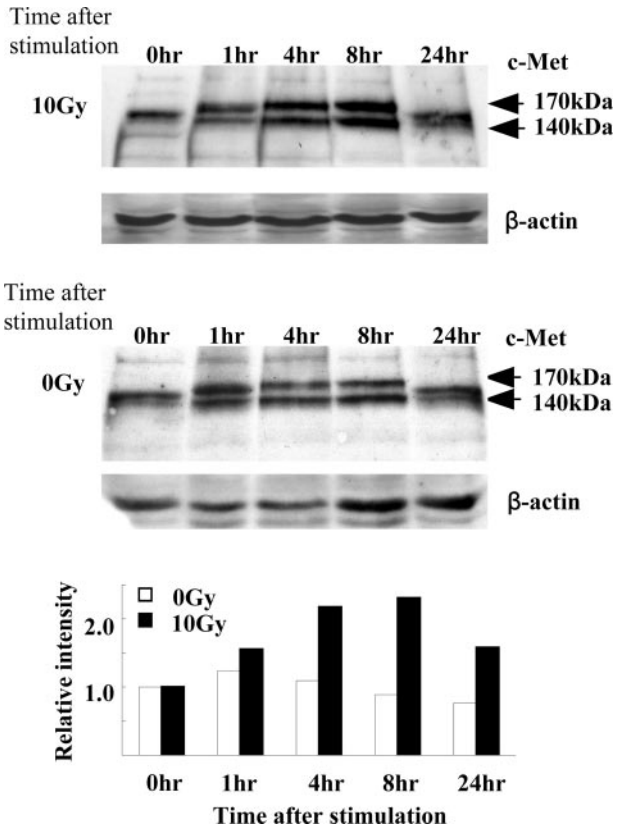

Fig. 5. Immunoblot analysis of c-Met expression in Suit-2 cells after exposure to the supernatants from either 10 Gy irradiated or nonirradiated MRC5 cells. The value in bar graph represents a band intensity of c-Met relative to the intensity at 0 h after normalization by the corresponding β-actin expression.

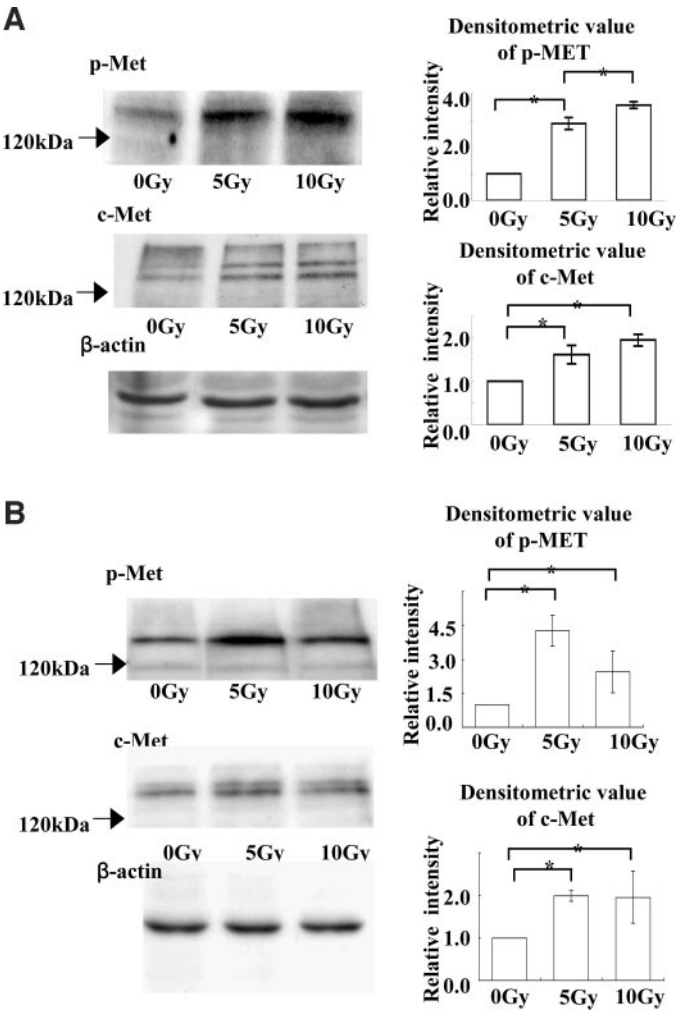

Fig. 6. Immunoblot analysis of c-Met and p-Met expression in Suit-2 cells at 8 h after exposure to the supernatant from either nonirradiated or irradiated (5 or 10 Gy) MRC5 cells (*A*) and pancreatic fibroblasts (*B*). The value in *bar graph* represents a band intensity of p-Met or c-Met (means and SD of three independent experiments) relative to the intensity at 0 Gy after normalization by the corresponding β-actin expression. *, $P < 0.01$

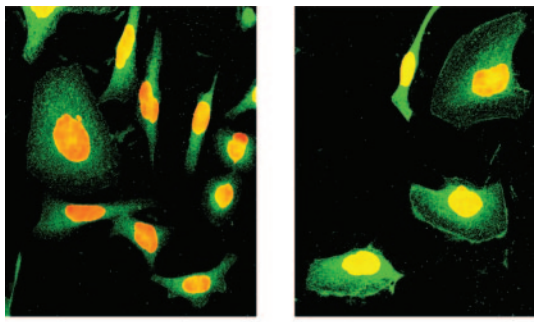

Fig. 7. Laser-scanning confocal microscopy of Suit-2 pancreatic cancer cells stained with an anti-c-Met antibody (*green*) showing an enhanced fluorescent intensity of c-Met on the cell membrane after exposure to the supernatant from 10 Gy-irradiated MRC5 cells.

NK4 Inhibits the Accelerated Invasiveness of Suit-2 Cells Cocultured with Irradiated Fibroblasts. NK4 is composed of 447 amino acids, which is an intramolecular region of HGF and includes a specific domain binding to c-Met receptor (36). We and other investigators (24, 25, 37, 38) have shown that NK4 acts as a specific

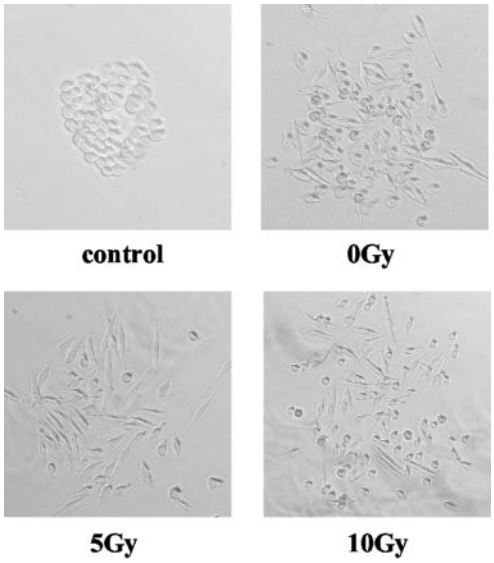

Fig. 8. Scattering of Suit-2 cells before and after exposure to the supernatant from nonirradiated (0 Gy) or irradiated MRC5 cells at 5 or 10 Gy.

HGF antagonist and inhibits the malignant behaviors of various cancer cells induced by the HGF/c-Met pathway. To further confirm the role of HGF/c-Met pathway in the increased invasiveness of pancreatic cancer cells cocultured with irradiated fibroblasts, we performed coculture invasion assay in the presence or absence of NK4 or anti-HGF antibody. The invasion of Suit-2 cells was accelerated when they were cocultured with irradiated fibroblasts, but the increased invasiveness was completely blocked in the presence of NK4 (at a concentration of 5 or 10 μg/ml; Fig. 9). The anti-HGF antibody showed the same inhibitory effect at almost the same dose as NK4 (data not shown). Furthermore, NK4 (10 μg/ml) inhibited the increased expression of c-Met/p-Met and the biphasic activation of MAPK in suit-2 cells exposed to the supernatant from irradiated fibroblasts (data not shown). These results indicate that HGF/c-Met pathway is a possible critical factor to induce invasiveness of Suit-2 cells in response to coculture with irradiated fibroblasts.

To rule out the effect of the change of basic FGF on phosphorylation of c-Met, we used the anti-FGF basic antibody in the same supernatant model and found that the anti-FGF antibody did not affect the phosphorylation of c-Met (data not shown).

The mRNA Expression of HGF and c-met in Cancer Cells and Fibroblasts. Although HGF is known to be secreted primarily from stromal cells (*e.g.,* fibroblasts), there is a possibility that HGF produced from cancer cells in an autocrine manner participates in the activation of HGF/c-Met and MAPK pathway and subsequent increase in the invasiveness of pancreatic cancer cells cocultured with irradiated fibroblasts. To rule out this possibility, we performed reverse transcription-PCR to examine the mRNA expression of HGF and c-Met in a panel of pancreatic cancer cell lines and fibroblasts. Reverse transcription-PCR showed no detectable expression of HGF in Suit-2 and Capan1, whereas the expression was detected in SW1990 and fibroblasts (MRC5 and primary pancreatic fibroblasts; Fig. 10). Expression of c-met mRNA was detectable in all of the cell lines and fibroblasts tested (Fig. 10). These results raise the possibility that autocrine loop of HGF might play a role in the increased invasiveness of SW1990 but not of Suit-2 and Capan-1 in response to coculture with irradiated fibroblasts.

Irradiated Fibroblasts Promote the Invasion of Pancreatic Cancer in *ex Vivo* Models. To support the *in vitro* data, we evaluated the effect of irradiated fibroblasts on the invasion of pancreatic cancers in *ex vivo* models. As shown in Fig. 11*A*, the tumor derived from Suit-2 cells and 10 Gy-irradiated MRC5 cells invaded into normal pancreatic tissues without forming a capsule, whereas the tumor cells derived from Suit-2 cells and nonirradiated MRC5 cells were encapsulated by multiple layers of stromal cells. Also, the same result was found in pancreatic fibroblasts. Histological estimation of tumor invasion indicated that the invasive score in groups with irradiated fibroblasts was significantly higher than that with nonirradiated fibroblasts (MRC5 and pancreatic fibroblasts; Fig. 11*B*; $P = 0.013$ and 0.038, respectively).

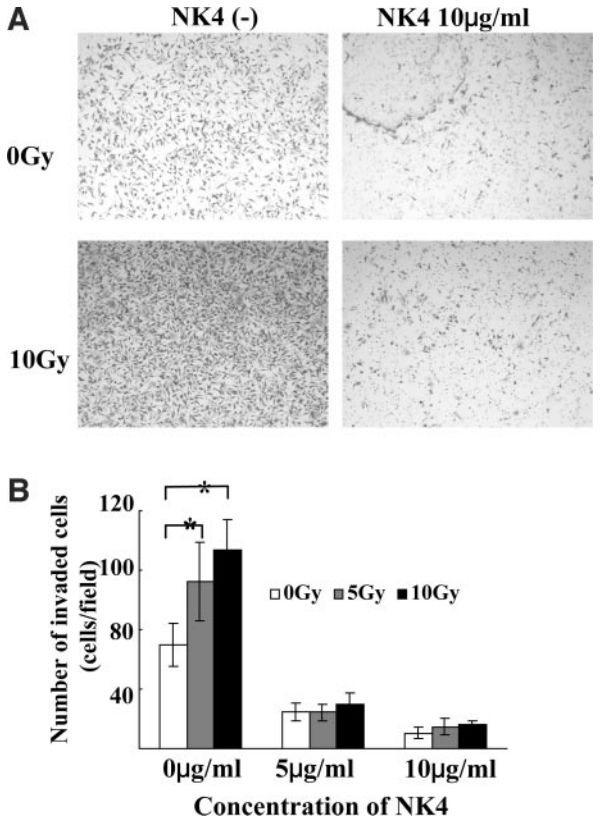

Fig. 9. NK4 inhibits the enhanced invasion of Suit-2 cells cocultured with irradiated MRC5 cells. Photomicrographs of *in vitro* invasion assay in Suit-2 cells cocultured with nonirradiated or 10-Gy irradiated MRC5 cells in the presence or absence of 10 μg/ml NK4. H&E stain, ×40. *A,* the number of invaded Suit-2 cells cocultured with MRC5 cells in the absence or presence of NK4 at a concentration of 5 or 10 μg/ml. *B,* each value represents the mean ± SD of triplicate measurements. *, $P < 0.01$

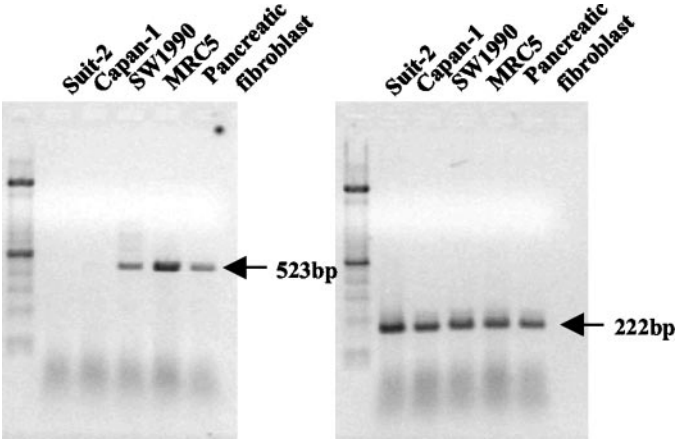

Fig. 10. Reverse transcription-PCR analysis of HGF and c-met mRNA expression in three pancreatic cancer cell lines (*Suit-2, Capan-1,* and *SW 1990*), *MRC5* fibroblasts, and primary *pancreatic fibroblasts*. The HGF expression is detectable as a 532-bp product in SW1990, MRC5, and pancreatic fibroblasts, whereas the c-met expression is detectable as a 222-bp product in all of the cell lines and fibroblasts tested.

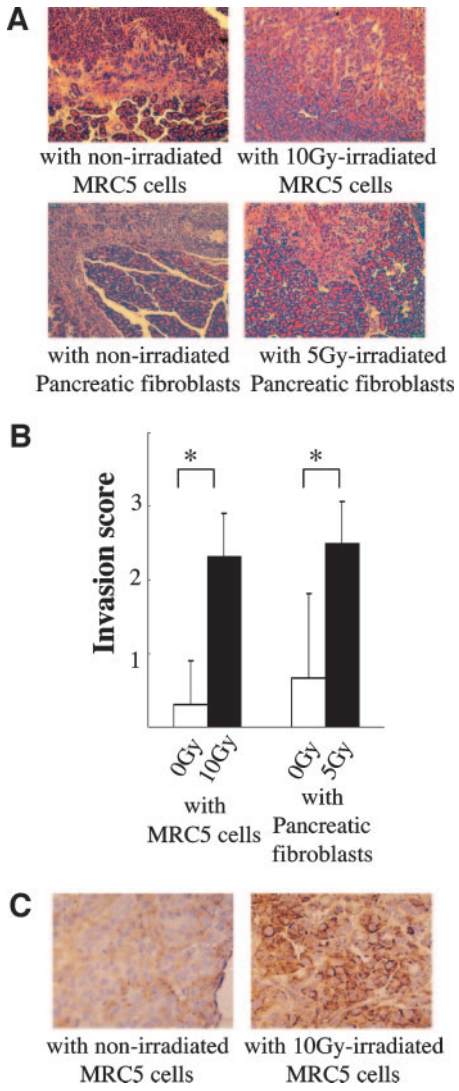

Fig. 11. MRC5 cells and pancreatic fibroblasts were irradiated at 10 or 5 Gy, respectively, just before implantation. Suit-2 cells (1×10^6) and 1×10^6 irradiated or nonirradiated fibroblasts were mixed well and implanted into the pancreas of mice. Three or four mice were used in each group. Seven days after implantation, tumors were resected with the surrounding tissues. Tissues were stained with H&E ×100 (*A*) or anti-c-Met phosphospecific antibodies ×400 (*C*) and evaluated for the invasiveness as described in "Materials and Methods" (*B*). *, $P < 0.01$

Furthermore, we evaluated the effect of irradiated fibroblasts on p-Met expression in tumors using immunohistochemical studies. As shown in Fig. 11*C,* clearly enhanced expression of p-Met was found in tumors with irradiated MRC5 cells as compared with that in tumors with nonirradiated MRC 5 cells. Also, similar results were observed in the same model using pancreatic fibroblasts.

DISCUSSION

Despite the fact that infiltrating adenocarcinoma of the pancreas is often characterized by an abundant desmoplastic stroma and that tumor-stromal interactions play a critical role in tumor invasion and metastasis, the relationship between radiation to stromal cells and the invasive phenotype of pancreatic cancer is unknown. In the present study, we demonstrate, for the first time, that coculture with irradiated fibroblasts significantly accelerates the invasion of certain pancreatic cancer cells through enhanced tumor-stromal interactions including activation of the HGF/c-Met system. Our present findings raise the possibility that radiation to stromal components as well as to neoplastic cells themselves could affect the progression of pancreatic cancer and therefore may have important clinical implications.

There are some clinical data suggesting that local radiation to tumors or normal tissue leads to the increase in metastasis. Fisher *et al.* (39) reviewed some prior clinical observation regarding enhanced metastasis after radiation therapy for breast cancer. Stjernsward (40) implicated routine postoperative radiation therapy as leading to increased mortality in early-stage cancers, presumably because of increased distant metastasis. Anderson *et al.* (41) demonstrated that local control failures after radiation therapy of cervix cancer was associated with increased distant metastasis. In addition, some investigators reported that metastasis occasionally appeared initially or preferentially in previously irradiated tissue in a various of tumors, such as melanoma (42), nasopharynx carcinoma (43), breast carcinoma (40), and uterine carcinoma (44).

Also, in experimental models, previous studies have implicated the relationship between radiation to the stromal tissues and the metastatic phenotype of malignant tumors. For example, radiation to the tumor bed stroma enhances the metastatic spread of carcinomas and sarcomas in mice (45). It has also been shown that metastasis of squamous cell carcinoma was more frequently observed in mice in which tumors developed in previously irradiated tissue than in mice with tumors in nonirradiated tissue (46). Our present study using *in vitro* coculture system and *ex vivo* models supports these *in vivo* findings and suggests that the accelerated metastasis in these irradiated tissues may be mediated by tumor-stromal interactions.

Recent evidence has shown that in several experimental systems, irradiation can promote invasion and metastasis of cancer cells by activating several genes/pathways important for tumor invasion and metastasis. For example, sublethal doses of irradiation enhance the migration and invasiveness of malignant glioma cells (9). In addition, radiation to a primary Lewis lung carcinoma results in accelerated growth of previously dormant lung metastases in mice (8). We have previously reported that radiation to pancreatic cancer cells enhances their invasive ability via up-regulated expression of c-Met and increased activity of MMP (10, 11). In the present study, we also observed that irradiation to pancreatic cancer cells resulted in an increase in their invasion ability when they were cocultured with nonirradiated fibroblasts, but the increase was modest. By contrast, the invasiveness of nonirradiated pancreatic cancer cells was markedly increased when they were cocultured with irradiated fibroblasts. These findings suggest that radiation to stromal fibroblasts could lead to an increased invasiveness of pancreatic cancer cells indirectly through enhanced tumor-stromal interactions even if the neoplastic cells themselves do not receive irradiation. In this respect, current radiation strategies (especially the setting of target radiation field) need to be carefully reevaluated.

To gain insights into the mechanisms by which coculture with irradiated fibroblasts increases the invasiveness of pancreatic cancer cells, we investigated the expression of HGF/c-Met system, one of the major pathways involved in tumor-stromal interactions, in our experimental models. We found that the increased invasiveness in response to coculture with irradiated fibroblasts was associated with phosphorylation of c-Met, possibly via increased expression of c-Met. These findings suggest that a soluble mediator(s) released from irradiated fibroblasts may trigger the activation of c-Met in pancreatic cancer cells. It has been shown that c-Met expression is induced by several growth factors and cytokines including HGF and basic-FGF (47, 48). In the present study, however, the secretion of HGF from fibroblasts was not increased after irradiation. We did observe an increase in the expression of basic FGF, a potent inducer of c-Met (49), in the supernatant from irradiated fibroblasts, although the concentration (~30 pg/ml) was insufficient to up-regulate p-Met because anti-FGF antibody did not inhibit the expression of p-Met. In addition, under the complete blockade of basic FGF, radi-

ation-enhanced invasion potential was maintained. These findings suggest that increased basic FGF is not a main factor responsible to induce enhancement of the invasion potential in coculture with irradiated fibroblasts, although it may be partially involved in the change of the invasion potential, being independent of phosphorylation of c-Met.

We also demonstrated that NK4, a specific antagonist of HGF, completely inhibited the increased invasiveness of pancreatic cancer cells cocultured with irradiated fibroblasts. Notably, NK4 blocked the increased expression of p-Met and activation of MAPK in Suit-2 cells exposed to the supernatant from irradiated fibroblasts. These results further support the involvement of the HGF/c-Met system in the enhanced invasiveness of pancreatic cancer cells induced by irradiated fibroblasts. We and other investigators (28, 38, 50–52) have shown that NK4 inhibits growth, invasion, and metastasis of a variety of human cancers *in vivo* as well as *in vitro*. Our present results further support the therapeutic efficacy of NK4 to inhibit the invasion of cancer cells and suggest that simultaneous administration of NK4 during radiotherapy could be a novel approach to improve the treatment efficacy for patients with pancreatic cancer.

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