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Involvement of Intracellular Expression of FGF12 in Radiation-Induced Apoptosis in Mast Cells

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Apoptosis/FGF12/Intracellular expression/Ionizing radiation/Mast cell.

Several fibroblast growth factors (FGFs) are able to reduce and improve radiation-induced tissue damage through the activation of surface fibroblast growth factor receptors (FGFRs). In contrast, some FGFs lack classical signal sequences, which play roles in the release of FGFs, and the intracellular function of these FGFs is not well clarified. In this study, we evaluated the transcript levels of 22 FGFs in a human mast cell line, HMC-1, using quantitative RT-PCR and found that FGF2 and FGF12 were expressed in HMC-1 cells. FGF12 not only lacks classical signal sequences but also fails to activate FGFRs. HMC-1 cells were transfected with an expression vector of FGF12 to clarify the intracellular function of FGF12 after irradiation. The overexpression of FGF12 in HMC-1 cells decreased ionizing radiation-induced apoptosis, and siRNA-mediated repression of FGF12 expression augmented apoptosis in HMC-1 cells. The overexpression of FGF12 strongly suppressed the marked augmentation of apoptosis induced by inhibition of the MEK/ERK pathway with PD98059. In contrast, the mitogen-activated protein kinase (MAPK) scaffold protein islet brain 2 (IB2), which was reported to bind to FGF12, did not interfere with the anti-apoptotic effect of FGF12. The expression of FGF12 transcripts was also detected in murine cultured mast cells derived from bone marrow or fetal skin. These findings suggest that FGF12 intracellularly suppresses radiation-induced apoptosis in mast cells independently of IB2.

INTRODUCTION

The fibroblast growth factor (FGF) family is currently thought to be composed of 22 members in humans.[1)] FGFs have important roles in numerous physiological and pathological events such as embryogenesis, angiogenesis, and wound repair[2–5)] Recently, it has been reported that several FGFs (FGF1,[6)] FGF2,[6–8)] FGF4,[9,10)] FGF7,[11,12)] FGF10,[13)] and FGF20[14,15)]) were able to inhibit radiation-induced tissue damage because they could inhibit radiation-induced apoptosis of epithelial cells and also protect the vasculature against irradiation.[6)]

Mast cells are distributed close to the mucosal and epithelial interfaces within environment such as the skin, gastrointestinal tract, and the respiratory system and are involved in health and disease through the immune response, inflammatory reaction, and the regeneration of tissues.[16,17)] They are also involved in radiation injury, and a number of mast cells have been found in irradiated lesions in the lungs and skin.[18–20)] Mast cells express various growth factors, such as transforming growth factor, granulocyte-macrophage colony stimulating factor (GM-CSF), platelet-derived growth factor (PDGF), and vascular endothelial growth factor (VEGF).[21)] In addition, FGF2, FGF7, and FGF10 are expressed in mast cell, and seem to contribute to wound healing and tissue homeostasis either through the synthesis and release of FGFs or via the stimulation of the release of growth factors by dermal fibroblasts.[22,23)] A human mast cell line (HMC-1) was established from the peripheral blood of a patient with mast cell leukemia and has been shown to possess many of the characteristics of immature mast cells.[24)] HMC-1 cells express multiple chemokines;[25)] therefore, this cell line should also be useful for beginning to examine the mast cell expression of human growth factors.

FGF12, initially designated FHF1, was identified by its sequence homology to known FGFs; it has a high degree of homology to FGF11 (FHF2), FGF13 (FHF3), and FGF14 (FHF4), with 58% to 71% amino acid sequence identity with

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these FGFs.[4] However, FGF12 shows less than 30% amino acid identity to other FGFs. Generally, many of the actions of FGFs occur through the activation of surface fibroblast growth factor receptors (FGFRs), whereas FGF12 does not activate any FGFRs, but FGF12 can bind to heparin with high affinity like other FGFs.[26] FGF12 has structural similarity with FGF1 and FGF2, in that it lacks a classical signal sequence and contains a nuclear localization signal (NLS), so that transfection of the *FGF12* gene into human embryonic kidney 293 cells resulted in the accumulation of FGF12 in the nucleus without any release from the cells.[4] In addition, receptor-bound FGF1 is able to be endocytosed to reach the nucleus via the presence of the NLS.[27] Moreover, FGF1 can interact with intracellular proteins such as FIBP, p34, CK2, and mortalin.[28–31] Therefore, the subsequent nuclear localization of FGF1 may play an important role as a component in signal transduction leading to proliferation.[32] Thus, we speculated that FGF12 might exert its effects inside the cells without any involvement of FGF receptors.

In this study, we investigated the expression of FGFs in HMC-1 cells, and found that FGF12 was expressed in HMC-1 cells, so we performed transfection of the *FGF12* gene into HMC-1 cells to investigate the intracellular effects of FGF12. As a result, the expression of the *FGF12* gene in HMC-1 cells was strongly involved in the repression of radiation-induced apoptosis. This study provides the first evidence that FGF12 intracellularly down-regulates radiation-induced apoptosis in a mast cell line.

MATERIALS AND METHODS

Cell culture, reagents, and irradiation

A human leukemic mast cell line (HMC-1) was a generous gift from Dr. J. H. Butterfield (Mayo Clinic, Rochester, MN, USA).[24] A human dermal fibroblast cell line, HFFF2, was obtained from the European Collection of Cell Cultures (Salisbury, UK). A human keratinocyte cell line, HaCaT, was generously provided by Dr. N. E. Fusenig (German Cancer Research Center, Heidelberg, Germany). A human gastric cancer cell line, MKN45, human colon adenocarcinoma cell lines (HT29, COLO201), and a murine mastocytoma cell line, P-815, were obtained from Health Science Research Resources Bank (Osaka, Japan). HMC-1 and P-815 were maintained in medium consisting of RPMI (Sigma, St Louis, MO, USA) supplemented with 10% heat-inactivated fetal calf serum (FCS). HFFF-2, HaCaT, MKN45, HT29, and COLO201 were maintained in medium consisting of DMEM (Gibco, Grand Island, NY, USA) supplemented with 10% heat-inactivated FCS. Anti-human FGF12 (P-12) polyclonal antibody was purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Anti-G3PDH polyclonal antibody (2275-PC-1) was obtained from Trevigen (Gaithersburg, MD, USA). Hoechst 33258 (Bisbenzimide H 33258 Fluorochrome, Trihydrochloride) was obtained from Calbiochem (La Jolla, CA, USA). LY294002, PD98059, and SB203580 (inhibitors of phosphatidylinositol-3 kinase (PI-3K), MEK, and p38 MAPK, respectively) were purchased from Calbiochem (La Jolla). The cells were irradiated with X-rays using an X-ray generator Pantak HF-320S (Shimazu, Kyoto, Japan) at a dose rate of approximately 2.4 Gy/min.

Transfection of FGF12 gene

The coding sequence of the human *FGF12* gene, which was provided in a Gateway entry vector, was purchased from Invitrogen (IOH35339). The insert was transferred into pcDNA3.2-DEST expression vector using LR clonase according to the GATEWAY Cloning Technology instruction manual (Invitrogen, Carlsbad, CA, USA), and then the *FGF12* gene sequence in the expression vector was confirmed by sequencing. HMC-1 cells were transfected with the expression vector using FuGENE6 lipofection reagent (Boehringer Mannheim Biochemicals, Indianapolis, IN, USA) and selected in the presence of 1.0 mg/ml geneticin (G418) (Gibco, Grand Island, USA) for 2–4 weeks to obtain stable transfectants. Limiting-dilution cloning was performed to stabilize and enhance the prevalence of transfectants with overexpression of FGF12, which was confirmed by competitive RT-PCR analysis and western blotting.

In vitro siRNA assay

Stealth RNAi is a type of chemically modified siRNA, which was obtained from Invitrogen. The synthesized oligonucleotides for the target site of each gene were as follows: FGF12, 5'-caa ggu uau uca gcc agc agg gau a-3' and 5'-uau ccc ugc ugg cug aau aac cuu g-3'; IB2, gac uuc ugg uuc cgu ggc uuc aac a-3' and 5'-ugu uga agc cac gga acc aga agu c-3'; and the negative control, 5'-caa uau uga cug acc gga cgg gau a-3' and 5'-uau ccc guc cgg uca guc aau auu g-3'. Stealth RNAi against the *FGF12* gene was conjugated with Alexa Fluor 546. Two complementary oligonucleotides were annealed and then modified chemically by the manufacturer. Each Stealth RNAi duplex was transfected at a final concentration of 50 nM using Lipofectamine™ RNAiMAX according to the manufacturer's protocol (Invitrogen). Following transfection, the cells were cultured for 48 h and then irradiated with X-rays. The cells were harvested for each assay at 24 h after irradiation. The silencing efficiency of siRNA was confirmed by competitive RT-PCR analysis at 24 and 48 h after siRNA transfection.

Competitive RT-PCR assay

A competitive RT-PCR assay was performed to determine the amount of transcripts of 22 human FGFs, IB2, and β-actin in each cell type as described previously.[33,34] The levels of murine Fgf2, Fgf7, and Fgf12 transcripts were also quantified by the same method. In brief, total RNA was isolated from the cells using an RNeasy Mini kit according to

the manufacturer's protocol (Qiagen, Hilden, Germany). Complementary DNAs (cDNAs) were synthesized with oligo(dT) primers from 1 μg of RNA in a total volume of 20 μl of reaction mixture using the Superscript III preamplification system (Invitrogen). DNA competitors of the appropriate sizes were generated using a Competitive DNA Construction kit (Takara Bio, Otsu, Japan). Competitive RT-PCR was performed using a Mastercycler (Eppendorf) in a total volume of 25 μl of reaction buffer containing 5 μl of standard DNA or sample cDNA, 5 μl of competitor DNA at the optimal concentration, each primer of the gene-specific primer set at 0.2 μM, and 0.5 U of ExTaq (Takara Bio, Otsu, Japan). The human representative primer sets were as follows: FGF2, 5'-cta acc gtt acc tgg cta tga agg-3' and 5'cca agt tta tac tgc cca gtt cgt-3'; FGF7, 5'-tgg atc ctg cca act ttg ctc tac-3' and 5'-cac aat tcc aac tgc cac tgt cct-3'; FGF10, 5'-ttt gtt gct gtt ctt ggt gtc ttc-3' and 5'-atc ctc tcc ttc agc tta cag tca-3'; FGF12, 5'-gac gaa aac agc gac tac act ctc-3' and 5'-cac aac ttt gcc tcc att cat ggt-3'; IB2, 5'-gga cga ctt ctg gtt ccg tgg ctt-3' and 5'-cgt acg cca ggt gct ctt ggt agt-3'; and human β-actin, 5'-gat atc gcc gcg ctc gtc gtc gac-3' and 5'-cag gaa gga agg ctg gaa gag tgc-3'. After competitive RT-PCR, 1 μl of PCR product was electrophoresed and analyzed with a DNA 1000 LabChip of a 2100 BioAnalyzer System (Agilent Technology, Palo Alto, CA, USA). The peak areas of the amplified fragments were automatically quantified using the BioAnalyzer to estimate the ratio of each gene to its competitor. The values for the transcripts were plotted on the respective standard curves to obtain the actual amount of each transcript. The actual amount of each gene transcript (copies/μl) was divided by that of β-actin ($\times 10^5$ copies/μl) for normalization (Table 1 and 2). Each normalized amount was further divided by that of the control sample to obtain the relative rate of expression (Fig. 1, 2 and 3).

Western blot assay

We determined the amounts of FGF12 in HMC-1 cells by western blot analysis. Cell pellets were lysed in an ice-cold solution containing 40 mM Tris-HCl (pH 8.0), 0.1% NP-40, 120 mM NaCl, and protease inhibitors, diethylenetriaminepentaacetic acid (DETAPAK) and protease inhibitor cocktail (Complete) (Roche Applied Science). Forty micrograms of proteins separated by 12% SDS-PAGE were transferred to a nitrocellulose membrane using a Mini Trans-Blot Electrophoretic Cell (Bio-Rad, Richmond, CA, USA). The membrane was blocked with PBS containing 5% skimmed milk at 4°C overnight, and then incubated with 1 μg/ml of anti-human FGF12 monoclonal antibody (P-12) or anti-G3PDH (diluted 1:5000). After incubation of the blot with HRP-conjugated second antibody, HRP on the blot was detected with ECL Plus Western Blot Detection Reagents (GE Healthcare, Little Chalfont Buckinghamshire, UK) as recommended by the manufacturer, and the signal was visualized using Fuji X-ray film.

Fluorescent staining of the nuclei with Hoechst 33258

The nuclei of apoptotic cells undergo condensation, which can be identified as an increase in the intensity of nuclear fluorescence stained with Hoechst 33258.[35] Radiation-induced apoptosis of HMC-1 cells was assessed by microscopic examination of nuclear morphology using Hoechst 33258, as described previously.[36] Briefly, cells were plated at a density of 3×10^5 cells per 3.5-cm dish and cultured for 24 h. The cellswere irradiated with X-rays at a dose of 10, 20, or 30 Gy and cultured for a further 24 h. The cells were fixed in 1% glutaraldehyde and the condensed chromatin of nuclei was visualized by staining with 0.1 mg/ml of Hoechst 33258 for 30 min. The number of apoptotic cells was counted using an IX70 inverted microscope (Olympus, Tokyo, Japan). The percentage of apoptotic cells was determined from the examination of 2,000 cells in 10 fields.

Flow cytometric assay

The effect of overexpression of FGF12 in HMC-1 cells was determined by dual staining with Annexin V-FITC and propidium iodide (PI) using an rhAnnexin V/FITC kit (Bender MedSystems, Vienna, Austria). Cells were irradiated with X-rays at a dose of 10, 20, or 30 Gy and cultured for 24 h. Annexin V-FITC and PI were added to the cellular suspension according to the instruction manual of the manufacturer, and a suspension of stained cells was subjected to FACSCalibur flow cytometry (BD Biosciences, San Jose, CA, USA) and at least 10,000 events were counted for each sample to discriminate intact cells (Annexin V–/PI–) from apoptotic cells (Annexin V+/PI–) and necrotic cells (Annexin V+/PI+).

Table 1. Expression levels of the transcripts of various FGFs in human cell lines

	HMC-1	FHHH-2	IMR90	HaCaT	MKN45	HT29	COLO201
FGF2	1.5 ± 0.25	78 ± 12	120 ± 16	3.8 ± 0.57	36 ± 5.3	0	0
FGF7	0	31 ± 2.0	12 ± 1.6	0	0	0	0
FGF10	0	0.17 ± 0.084	0	0	0	0	0
FGF12	6.4 ± 0.053	0.072 ± 0.10	0	1.6 ± 0.028	3.8 ± 0.50	2.0 ± 0.092	0.29 ± 0.040

The amount of each FGF transcript (copies/μl) was normalized by that of β-actin transcripts ($\times 10^5$ copies/μl).

Table 2. Expression levels of the transcripts of various FGFs in murine fatal skin-derived cultured mast cells (FSMC), a murine bone marrow-derived mast cells (BMMC), and mast cell line (P-815)

	FSMC	BMMC	P-815
FGF2	290 ± 110	79 ± 22	0
FGF7	300 ± 190	82 ± 2.8	0
FGF12	11 ± 0.65	1.6 ± 0.51	0

The amount of each Fgf transcript (copies/μl) was normalized by that of β-actin transcripts ($\times 10^5$ copies/μl).

Preparation of mast cells

Murine fetal skin-derived cultured mast cells (FSMC) were prepared from day 14 fetal skin of Balb/c mice as reported previously.[37] Briefly, cells were separated from peeled fetal skin specimens with 0.25% trypsin (Gibco, Grand Island) in HBSS containing Ca^{2+} (Gibco, Grand Island) for 20 min at 37°C. The cell suspension was plated at the density of 5×10^4 cells/ml in complete RPMI medium with 10% FCS, 10 ng/ml of recombinant IL-3 and 10 ng/ml of recombinant stem cell factor and cultured for 14 days at 37°C in a 5% CO_2 incubator. Nonadherent cells were collected by pipetting gently and subjected to density gradient centrifugation in 1.055 g/ml Percoll (Biochrom AG, Berlin, Germany) to enrich the mast cell population. More than 95% purification of mast cells was confirmed by Toluidine Blue staining. Murine bone marrow-derived cultured mast cells (BMMC) were also prepared from bone marrow cell suspensions separated from femurs of BALB/c mice, as reported previously.[37]

RESULTS

FGF12 is expressed in HMC-1 cells

We evaluated the transcript levels of 22 FGFs in a human mast cell line, HMC-1, using competitive RT-PCR, and found that FGF12 mRNA was expressed in HMC-1 cells (Table 1). Previous studies have shown that FGF2, FGF7, and FGF10 are expressed in mast cells.[22,23] We found that FGF2 was expressed in HMC-1 cells, but expression of FGF7 and FGF10 was not detected in these cells. In addition, FGF12 was also expressed in a human gastric cancer cell line (MKN45), a human keratinocyte cell line (HaCaT), and human colon adenocarcinoma cell lines (HT29, COLO201), whereas human fibroblast cell lines (HFFF-2 and IMR90) expressed only low levels of FGF12 transcripts. However, these fibroblast cell lines expressed enormous amounts of FGF2 and FGF7 transcripts producing levels of 12 to 120 in our RT-PCR assay system. In contrast, the levels of the transcripts of FGF2 and FGF12 in HMC-1 cells were low, 1.5 and 6.4, respectively, in our assay systems. HMC-1 cells did not express transcripts of the other 20 FGFs (data not shown).

To examine the effects of ionizing radiation on the expression of FGF12, we determined the level of FGF12 expression in irradiated HMC-1 cells. HMC-1 cells were plated at a density of 3×10^5 cells per 3.5-cm dish in 2 ml of growth medium 24 h before irradiation. The cells were irradiated with X-rays at a dose of 10, 20, or 30 Gy and then cultured for a further 24 h. Competitive RT-PCR analysis showed that irradiation slightly increased the level of FGF12 transcripts in HMC-1 cells in a dose-dependent manner (Fig. 1A).

Anti-apoptotic effect of FGF12 gene overexpression in HMC-1 cells

HMC-1 cells were transfected with an expression vector containing the coding sequence of FGF12 in order to investigate the role of FGF12 in radiation-induced cell damage. As a control, the cells were transfected with pcDNA3 vector. We obtained ten clones of FGF12 transfectants, which had about 5-fold higher levels of FGF12 transcripts than the control cells (Fig. 1B). Each lysate was electrophoresed on a 12% SDS-polyacrylamide gel and subjected to western blot analysis using 1 μg/ml of anti-human FGF12 antibody (P-12). The FGF12 band was stained more strongly in FGF12 transfectants than in the control cells (Fig. 1C). We also confirmed the same total protein content of each sample by western blot analysis using anti-G3PDH antibody.

Each HMC-1 transfectant was spread at an initial concentration of 3×10^5 cells per 3.5-cm dish and irradiated at a dose of 10, 20, or 30 Gy of X-rays. At 24 h after irradiation, apoptosis was determined by Hoechst staining (Fig. 1D). Irradiation increased the apoptosis of HMC-1-pcDNA3 cells, but not always in a radiation dose-dependent manner. The maximum number of apoptotic cells was seen at 20 Gy of irradiation, and the apoptotic cell number at 30 Gy tended to be lower than that at 20 Gy. The apoptotic cell number of HMC-1-pcDNA3 cells at 20 Gy was approximately 2-fold higher than that in the non-irradiated cells. In contrast, irradiation only slightly increased apoptosis in HMC-1-FGF12 cells in a dose-dependent manner, although the percentage of apoptotic cells in the FGF12 transfectants was very low (1.5% to 3%). The cells were subjected to two-color flow cytometric analysis to determine the levels of apoptosis using Annexin V-FITC 24 h after irradiation. FACSCalibur flow cytometry could discriminate apoptotic cells (Annexin V+/PI–) from the intact or necrotic cells. Irradiation increased apoptosis in HMC-1-pcDNA3 cells in a dose-dependent manner, and the apoptotic cell number was approximately 2.5-fold higher than that in non-irradiated cells (Fig. 1E). In contrast, the FGF12 transfectants showed a low frequency of apoptosis, and the frequency remained at approximately 2% even after irradiation. However, HMC-1-FGF12 cells showed almost the same percentage necrotic cell population (Annexin V+/PI+) as HMC-1-pcDNA cells

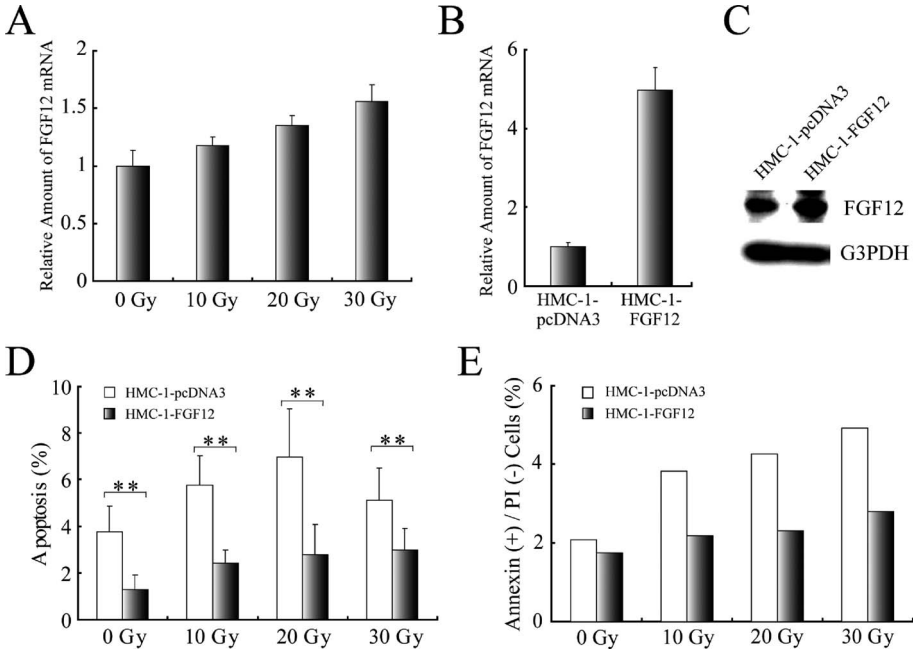

Fig. 1. Inhibition of radiation-induced apoptosis in HMC-1 cells overexpressing FGF12. HMC-1 cells were transfected with the control vector (HMC-1-pcDNA3) or the expression vector of FGF12 (HMC-1-FGF12). Stable transfectants were obtained by G418 selection and cloned by the limiting-dilution method. (A) HMC-1 cells were irradiated with X-rays at a dose of 10, 20, or 30 Gy. The level of FGF12 transcripts was measured by competitive RT-PCR 24 h after irradiation. The absolute amount of each transcript (copies/μl) was normalized by the amount of β-actin (copies/μl), and each normalized value was further calculated as the value relative to that in non-irradiated cells. (B) FGF12-transfectant cells were examined to determine the expression level of FGF12 transcripts by competitive RT-PCR. Each normalized value was further calculated as the value relative to that in HMC-1-pcDNA3 cells. (C) Each lysate was electrophoresed on a 12% SDS-polyacrylamide gel and subjected to western blot analysis using 1 μg/ml of anti-human FGF12 antibody (P-12). (D) The condensation of apoptotic cell nuclei was examined by the staining of cells with Hoechst 33258 at 24 h after X-ray irradiation at 10, 20, or 30 Gy in five independent experiments. The percentage of apoptotic cells was determined from the examination of 2,000 cells in 10 fields using a fluorescence microscope. Values are the mean ± SD. $^{**}P < 0.01$. (E) Each transfectant was stained with propidium iodide (PI) and Annexin V-FITC using an rhAnnexin V/FITC kit 24 h after X-ray irradiation at 10, 20, or 30 Gy in two independent experiments. The cells were subjected to two-color flow cytometric analysis to determine the levels of apoptosis (Annexin V+/PI–).

after irradiation (data not shown). Therefore, FGF12 overexpression did not increase the necrotic cell number of HMC-1 cells. In conclusion, the overexpression of FGF12 in HMC-1 cells significantly decreased radiation-induced apoptosis.

Repression of FGF12 in HMC-1 cells

HMC-1 cells were transfected with Stealth RNAi targeted against the *FGF12* gene (FGF12 siRNA) or with the negative control Stealth RNAi (Control siRNA) (Fig. 2). These molecules were conjugated with orange fluorescent Alexa Fluor 546 so that transfection could be assessed by flow cytometry. The transfection efficiency ranged from 75.3% to 90.6% and the average efficiency was 86.8% 48 h after transfection (data not shown). A competitive RT-PCR assay showed that introduction of FGF12-targeted Stealth RNAi into HMC-1 cells reduced the level of FGF12 mRNA by approximately 90% compared with that in HMC-1 cells (Fig. 2A). Transient transfection of the control siRNA by itself did not affect spontaneous or radiation-induced apoptosis (data not shown). Each HMC-1 transfectant was irradiated with X-rays at a dose of 10, 20, or 30 Gy at 48 h after transfection, and then apoptosis of the cells was determined by Hoechst staining (Fig. 2B). Without any irradiation, the percentage of apoptotic cells among both FGF12 siRNA cells and the control siRNA cells remained at the same level (about 2%). However, repression of FGF12 in HMC-1 cells significantly increased the population of apoptotic cells in a manner dependent on the dose of irradiation. The apoptotic cell number was significantly higher in FGF12 siRNA cells than in the control cells at 20 or 30 Gy of irradiation ($P < 0.01$). After 30 Gy of irradiation, about 5% of the FGF12

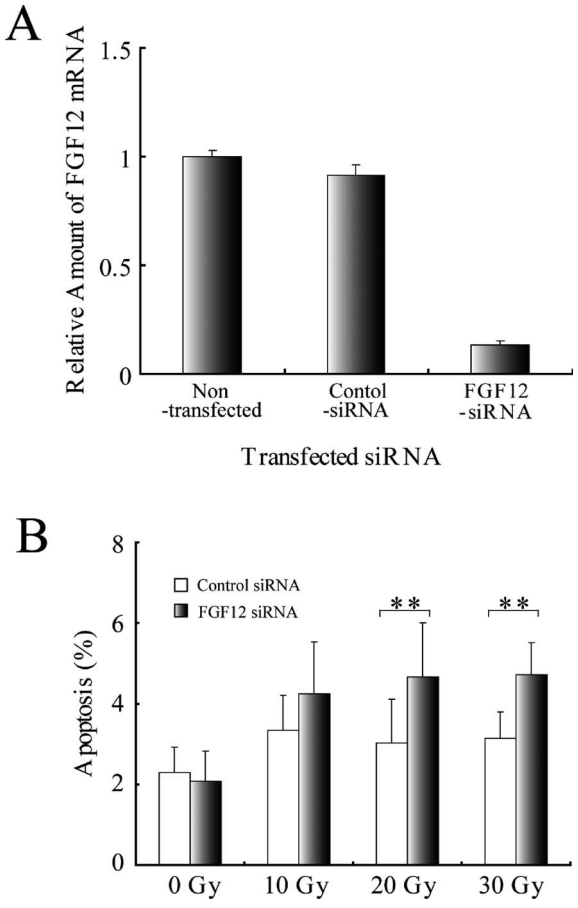

Fig. 2. Increase of radiation-induced apoptosis in HMC-1 cells by repression of FGF12. HMC-1 cells were transfected with Alexa 546-conjugated Stealth RNAi targeting the *FGF12* gene (FGF12 siRNA) or Alexa 546-conjugated control Stealth RNAi (Control siRNA). (A) The level of FGF12 transcripts was quantified by competitive RT-PCR in HMC-1 cells 48 h after transfection. The absolute amount of each transcript (copies/μl) was normalized by the amount of β-actin (copies/μl), and the average value of the normalized amount was further normalized by the value in non-transfected HMC-1 cells (Non-transfected). (B) The transfected cells were cultured in RPMI with 10% FCS for 48 h after transfection and then irradiated with X-rays at a dose of 10, 20, or 30 Gy. The cells were stained with Hoechst 33258 at 24 h after irradiation to estimate the induction of apoptosis by irradiation. Values are the mean ± SD. $^{**}P < 0.01$.

siRNA transfectants were apoptotic, whereas about 3% of the cells were apoptotic in the control transfectant cells at this dose of irradiation.

Involvement of IB2 in radiation-induced apoptosis

To examine the effects of the mitogen-activated protein kinase (MAPK) scaffold protein islet brain 2 (IB2) in radiation-induced apoptosis, HMC-1 cells were transfected with Stealth RNAi targeted against the *IB2* gene (IB2 siRNA) or with the negative control Stealth RNAi (Control siRNA). A competitive RT-PCR assay showed that introduction of IB2-targeted Stealth RNAi into HMC-1 cells reduced the level of IB2 mRNA by approximately 80% compared with that in non-transfected HMC-1 cells (Fig. 3A). Each HMC-1 transfectant was irradiated with X-rays at a dose of 10, 20, or 30 Gy 48 h after transfection, and then apoptosis of the cells was determined by Hoechst staining (Fig. 3B). Repression of IB2 in HMC-1 cells significantly increased the population of apoptotic cells even without any irradiation. Irradiation progressively increased the percentage of apoptotic siRNA IB2 cells to between 4% and 10% in a dose-dependent manner, and at all the tested doses, the apoptotic cell number was significantly higher in siRNA IB2 cells than in the control siRNA cells. Thus, the repression of IB2 expression in HMC-1 cells significantly increased radiation-induced apoptosis.

Limited involvement of IB2 in anti-apoptotic effect of FGF12 in HMC-1 cells

To examine the possible involvement of IB2 in the anti-apoptotic effect of FGF12, IB2 expression was repressed in FGF12 transfectants using siRNA transfection targeted against the *IB2* gene (IB2 siRNA) (Fig. 3C). Each HMC-1 transfectant was irradiated with X-rays at a dose of 10, 20, or 30 Gy at 48 h after siRNA transfection, and then apoptosis of the cells was assessed by Hoechst staining 24 h after irradiation. Repression of IB2 in HMC-1-FGF12 cells significantly increased the population of apoptotic cells even without any irradiation; however, irradiation did not increase the percentage of apoptotic cells of IB2-repressed HMC-1-FGF12 cells (HMC-1-FGF12 IB2 siRNA). On the other hand, irradiation progressively increased the percentage of apoptotic cells in IB2-repressed mock transfectants in a dose-dependent manner (HMC-1-pcDNA3 IB2 siRNA). At all the tested doses of irradiation, the apoptotic cell number was significantly higher in IB2-repressed mock transfectants (HMC-1-pcDNA3 IB2 siRNA) than in IB2-repressed FGF12 transfectants (HMC-1-FGF12 IB2 siRNA). These findings indicated that overexpression of FGF12 could overcome the effect of repression of IB2, so that HMC-1-FGF12 transfectant cells did not show an increase of radiation-induced apoptosis after the repression of IB2. This finding suggested that the anti-apoptotic effect of FGF12 was independent of IB2.

No effect of inhibitors of PI-3K, MEK, and p38 MAPK on the anti-apoptotic effect of overexpression of FGF12

To analyze the anti-apoptotic signaling pathway of FGF12, inhibition of PI-3K, MEK, or p38 MAPK in HMC-1 transfectant cells was performed using appropriate inhibitors (LY294002, PD98059, and SB203580, respectively) in irradiated HMC-1 cells (Fig. 4). Apoptosis of HMC-1 cells was assessed by microscopic examination of nuclear mor-

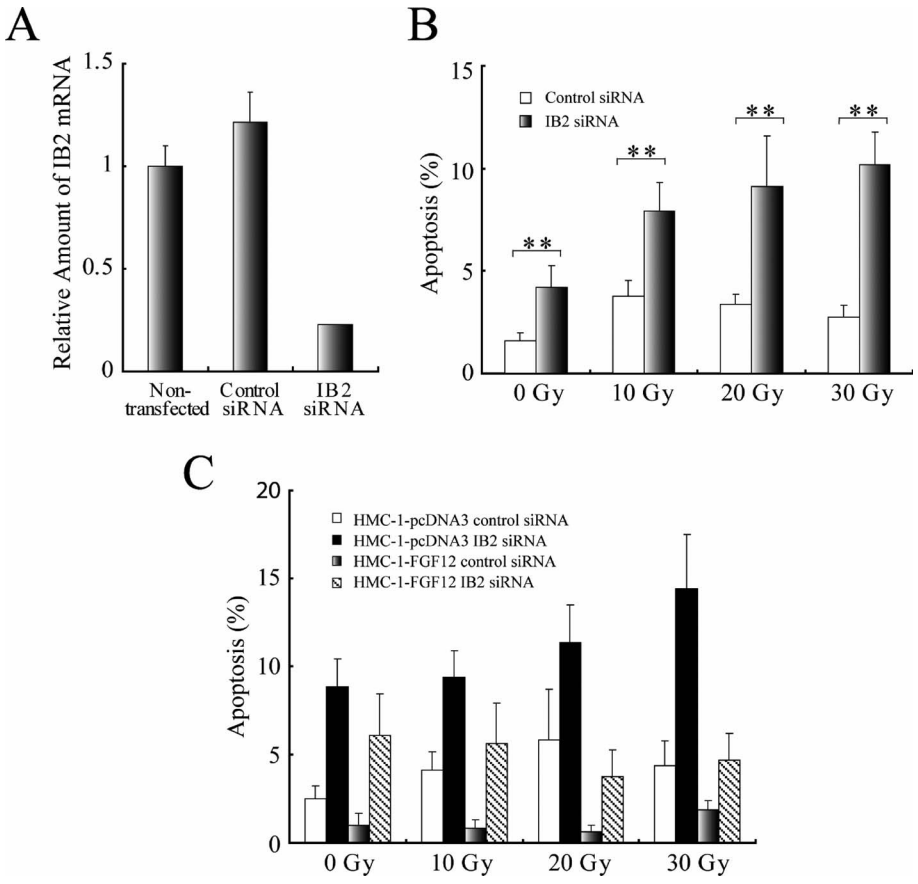

Fig. 3. Limited involvement of IB2 in the anti-apoptotic effect of FGF12 in HMC-1 cells. (A) HMC-1 cells were transfected with Stealth RNAi targeting the *IB2* gene (IB2 siRNA) or the negative control Stealth RNAi (Control siRNA). The level of IB2 transcripts was quantified by competitive RT-PCR in HMC-1 cells 48 h after transfection. The amount relative to that in non-transfected HMC-1 cells (Non-transfected) was determined as above. (B) Each transfectant was irradiated at 10, 20, or 30 Gy 48 h after transfection, and the level of apoptosis was estimated by Hoechst 33258 staining at 24 h after X-ray irradiation. Values are the mean ± SD. $^{**}P < 0.01$. (C) HMC-1 cells were transfected with the control vector (HMC-1-pcDNA3) or FGF12 (HMC-1-FGF12). The stable transfectant clones were transiently transfected with Stealth RNAi targeting the *IB2* gene (IB2 siRNA) or the negative control Stealth RNAi (control siRNA) and cultured in RPMI with 10% FCS for 48 h after transfection. Each transfectant was stained with Hoechst 33258 at 24 h after X-ray irradiation at 10, 20, or 30 Gy. Values are the mean ± SD.

phology using Hoechst 33258. Inhibition of MEK prominently induced apoptosis in HMC-1 mock transfectant cells, and the level of spontaneous apoptosis with PD98059 treatment was about six times that without treatment. Irradiation progressively increased the percentage of apoptotic cells among the mock transfectant cells, and maximal induction of apoptosis was attained at 20 Gy; however, overexpression of FGF12 inhibited the increase of apoptosis in the HMC-1 cells treated with PD98059. Inhibition of p38 MAPK with SB203580 in HMC-1 cells augmented radiation-induced apoptosis, but overexpression of FGF12 also inhibited the increase of apoptosis in the HMC-1 cells treated with SB203590. Inhibition of PI-3K increased spontaneous apoptosis in HMC-1 cells, but irradiation did not influence apoptosis in cells treated with LY294002. None of these inhibitors (LY294002, PD98059, or SB203580) significantly blocked the suppression of radiation-induced apoptosis by overexpression of FGF12 in HMC-1 cells.

FGF12 is expressed in murine cultured mast cells

We evaluated the transcript levels of Fgf2, Fgf7, and Fgf12 in murine fetal skin-derived cultured mast cells (FSMC) and murine bone marrow-derived cultured mast cells (BMMC) using competitive RT-PCR (Table 2). These cultured mast cells expressed enormous amounts of Fgf2 and Fgf7 transcripts (80 to 300 in our assay system); however, the level of Fgf12 transcripts in the cultured mast cells was much lower than that of Fgf2 or Fgf7 and the normalized amounts of Fgf12 transcripts were 11 and 1.6 in FSMC and BMMC, respectively. In contrast, none of Fgf2, Fgf7, or

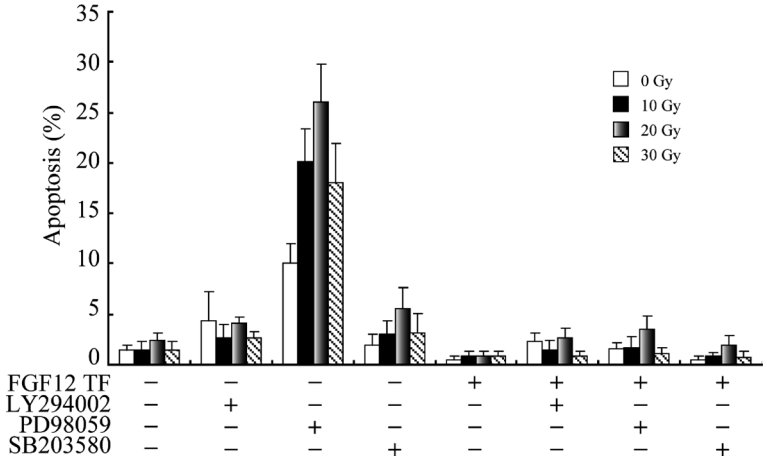

Fig. 4. Treatment of HMC-1-FGF12 cells with inhibitors of PI-3K, MEK, and p38 MAPK. HMC-1 cells were transfected with the control vector (FGF12 TF –) or the expression vector of FGF12 (FGF12 TF +). Stable transfectant clones were cultured in complete culture medium with 10 μM LY294002, 50 μM PD98059, or 50 μM SB203580 for 1 h and then irradiated with X-rays at a dose of 10, 20, or 30 Gy. The cells were stained with Hoechst 33258 at 24 h after irradiation to assess the induction of apoptosis by irradiation. Values are the mean ± SD.

Fgf12 was detected in the murine mastocytoma cell line P-815. Thus, expression of FGF12 might not be specifically observed in leukemic cell lines, but rather FGF12 might be constitutively expressed in normal mast cells.

DISCUSSION

FGF12 is a member of the FGF family, which contains 22 members in humans,[1] and is expressed in the developing and adult nervous systems, suggesting that it is related to nervous system development and function.[4] FGF12 is also expressed in the developing soft connective tissue of the limb skeleton and in presumptive connective tissue linking vertebrae and ribs.[38] However, there have been no reports showing that FGF12 is expressed in hematopoietic cell lines. We examined the expression of FGF12 in human cell lines, and found out that FGF12 was expressed in human leukemic mast cell line HMC-1, keratinocyte cell line HaCaT, gastric cancer cell line MKN45, and colon cancer cell lines (HT29 and Colo201), in addition to fibroblast cell lines, although the level of FGF12 expression was very low in all of these cells except HMC-1 cells. Recently, it was reported that the expression of FGF12 could be induced in human umbilical vein endothelial cell lines (HUVECs) by co-culturing with U87 human glioma cells.[39] These findings suggested that FGF12 was involved in not only the nervous system but also in various other tissues.

Mast cells are mainly composed of two kinds of cells, connective tissue mast cells (CTMC) and mucosal mast cells (MMC). They exist in different tissues: CTMC are distributed in the skin and peritoneal cavity, whereas MMC are situated in the mucosa of the bronchial tube and the gastrointestinal tract. We examined the level of Fgf12 transcripts in murine fetal skin-derived cultured mast cells (FSMC) and murine bone marrow-derived cultured mast cells (BMMC), which were cultured *in vitro* because FSMC exhibit important features of CTMC and BMMC have features of MMC.[37,40] The results showed that Fgf12 transcripts were expressed in both FSMC and BMMC, although BMMC expressed a relatively low level of Fgf12. However, Fgf12 was not detected in the murine mastocytoma cell line P-815, and Fgf12 was not specifically expressed in mast cell tumors. FGF12 protein might instead be expressed and play a physiological role in normal mast cells.

In this study, the transfection of the *FGF12* gene into HMC-1 cells was performed to investigate the effects of FGF12 on radiation damage, because we speculated that FGF12 might exert its effects inside the cells without any involvement of FGF receptors. HMC-1 cells were very resistant to radiation-induced apoptosis, and we found that 10 to 30 Gy was the most appropriate dose for evaluating radiation-induced apoptosis in HMC-1 cells by microscopic studies of nuclear morphology employing Hoechst 33258 staining. The number of apoptotic HMC-1 cells reached a maximum level after irradiation at 20 Gy, and the percentage of apoptotic HMC-1 cells was lower at 30 Gy than that at 10 or 20 Gy. Radiation-induced apoptosis in HMC-1 cells might have been attenuated by the induction of anti-apoptotic machinery. For example, irradiation slightly increased the level of FGF12 in HMC-1 cells in a dose-dependent manner (Fig. 1A). Therefore, induction of FGF12 may have inhibited apoptosis in the cells irradiated at a dose of 30 Gy.

Radiation-induced apoptosis was inhibited by the overexpression of FGF12 and promoted by the repression of FGF12 in HMC-1 cells. These results show that FGF12 intracellularly down-regulates radiation-induced apoptosis because FGF12 was not released from the cells and does not activate any FGFRs. FGF12 can bind to islet-brain 2 (IB2), which is expressed in the brain, pancreas, and specific cell lines,[41,42] and the interaction of FGF12 with IB2 facilitates the association with and subsequent activation of p38δ.[43] It has been reported that p38 MAPK is activated by ionizing radiation,[44] and that activation of p38 MAPK can lead to the phosphorylation of a number of downstream targets, including p53.[45,46] Therefore, inhibition of p38 MAPK effectively attenuated radiation-induced apoptosis.[47] HMC-1 cells expressed not only FGF12 but also IB2 (Fig. 3); therefore, we speculated that FGF12 might act coordinately with the p38 MAPK signaling pathway to induce apoptosis. However, HMC-1 cells were resistant to radiation-induced apoptosis, and repression of FGF12 resulted in a dose-dependent increase in radiation-induced apoptosis of HMC-1 cells (Fig. 2B).

In addition, repression of IB2 also resulted in a prominent increase in spontaneous apoptosis of HMC-1 cells (Fig. 3) and inhibition of p38 MAPK with SB203580 in HMC-1 cells did not attenuate radiation-induced apoptosis (Fig. 4). Surprisingly, the overexpression of FGF12 significantly inhibited the increase of apoptosis induced by repression of IB2 in HMC-1 cells, although spontaneous apoptosis was increased in these cells owing to repression of IB2 (Fig. 3C). Therefore, we have no evidence that IB2 could function in association with the p38 MAPK signaling pathway to attenuate apoptosis. Accordingly, these findings suggested that FGF12 suppressed radiation-induced apoptosis in HMC-1 cells without the involvement of IB2.

On the other hand, inhibition of MEK with PD98059 in HMC-1 cells prominently induced apoptosis (Fig. 4). The MEK/ERK pathway plays an important role in cell survival and proliferation via growth factor activation.[48] Therefore, the MEK/ERK pathway might have a strong effect on the inhibition of apoptosis in HMC-1 cells. Indeed, siRNA-mediated repression of FGF12 resulted in a relatively slight increase of apoptosis, suggesting that other anti-apoptotic mechanisms, such as the IB2 or ERK pathway, compensated for the anti-apoptotic effect of FGF12. Inhibition of PI-3K, MEK, or p38 MAPK did not cancel the anti-apoptotic effect of overexpressed FGF12, indicating that FGF12 did not act upstream of these signaling molecules as an anti-apoptotic factor. In addition, the overexpression of FGF12 suppressed the drastic increase of apoptosis induced by MEK inhibitors in HMC-1 cells, suggesting that FGF12 was associated with the ERK pathway.

Accordingly, we obtained the interesting result that FGF12 acted as an anti-apoptotic factor that could overcome radiation-induced tissue damage. In addition, our findings revealed a novel function of intracellular FGFs such as FGF12, whose actions might be different from those of FGFR-mediated signaling pathways, because they contain a nuclear localization signal (NLS) without a classical signal sequence, such as FGF1 and FGF2. Therefore, further investigation of FGF12 could lead to further understanding of FGFs and may lead to substantial benefits for patients with radiation injuries or cancer treatment using ionizing radiation.

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