NF-κB decoy potentiates the effects of radiation on vascular smooth muscle cells by enhancing apoptosis

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Accepted 12 January 2005

Abstract

NF-κB promotes cell survival against external stress such as radiation. We examined whether NF-κB decoy transfection enhances the antiproliferative effects of radiation on vascular smooth muscle cells (VSMCs) *in vitro*. The irradiation induced activation or nuclear translocation of NF-κB p65 in VSMCs was confirmed by immunofluorescence. NF-κB decoy transfection resulted in inhibition of the radiation-induced NF-κB activation in VSMCs and the subsequent reduction of transcription and translocation of ICAM, iNOS, and TNF-α, downstream molecules under the control of NF-κB. By using MTT assay, NF-κB decoy augmented the antiproliferative effects of radiation, where the effect of low dose radiation (2 and 8-Gy) of the cells transfected with NF-κB decoy was equivalent to the high dose (16-Gy) irradiated non-transfected cells at 48 h after irradiation: 1.06 ± 0.16, 1.11 ± 0.22, 1.20 ± 0.25, respectively. The decrease in proliferation and survival of the radiation treated cells by flow cytometry analysis showed that NF-κB inhibition did not show any additive effects on the cell cycle of the irradiated VSMCs, while apoptosis was significantly increased after NF-κB decoy transfection in the irradiated VSMCs (apoptosis fraction: $13.33 \pm 2.08\%$ vs. $26.29 \pm 7.43\%$, for radiation only vs. radiation+NF-κB decoy transfection, $P < 0.05$). In addition, at 48 h, NF-κB decoy transfection dose dependently (10 μM vs. 20 μM) inhibited proliferation of 16Gy-irradiated VSMCs, and showed greater antiproliferative efficacy than 100 μM sulfasalazine, a specific NF-κB inhibitor. These results indicate that NF-κB inhibition reduces proliferation and survival of irradiated VSMCs, likely by increased apoptosis rather than additive cell cycle arrest and suggest the possibility of adjunctive gene therapy using NF-κB decoy to improve efficacy and to decrease the adverse effects of intracoronary radiation therapy.

Keywords: gene therapy; NF-κB; radiation; vascular smooth muscle cells

Introduction

Restenosis is one of the most important problems in coronary angioplasty, and its major mechanisms are vascular remodeling and neointimal proliferation (Rajagopal and Rockson, 2003). Although the stent has significantly improved problems associated with negative remodeling, neointimal proliferation has still remained a big challenge for the clinician. Therefore, the inhibition of vascular smooth muscle cell (VSMC) proliferation, which constitutes the major portion of the neointima, is the main target of therapy in restenosis prevention. Accordingly, intracoronary radiation therapy (or coronary brachytherapy), is one of the most promising anti-restenotic modalities due to its antiproliferative, proapoptotic effects along with cell cycle arrest (Teirstein *et al.,* 1997). However, VSMCs are relatively resistant to radiation (Brenner *et al.,* 1996) compared with other cells and intracoronary radiation has been shown to have some adverse side effects at high doses such as late thrombosis and coronary artery aneurysm (Condado *et al.,* 1999; Farb *et al.,* 2003). Therefore, the development of adjunctive therapy is necessary, both to enhance the antiproliferative effects, and to decrease the dose and subsequent adverse effects of coronary brachytherapy.

Exposure of cells to radiation activates different kinds of transcriptional factors such as NF-κB and

p53 (Li and Karin, 1998; Jonathan *et al.,* 1999). Radiation is known to activate NF-κB through IKK (IκB kinase) activation (Li and Karin, 1998). Although NF-κB was initially considered as a pro-apoptotic factor because of its rapid activation in response to apoptotic signals and its involvement in the expression of apoptotic genes, such as TNF-α and c-myc, recent work have revealed an anti-apoptotic effect of NF-κB in response to a variety of apoptotic stimuli (Chen *et al.,* 2001) including radiation. Therefore, we postulated that the blocking of NF-κB might potentiate the antiproliferative effects of irradiation in VSMCs. This *in vitro* study was performed to elucidate the additive effects of NF-κB inhibition on the proliferation and apoptosis of irradiated VSMCs, and its effect on the cell cycle.

Materials and Methods

Cell culture

VSMCs were isolated enzymatically from thoracic aorta of male 6-week-old Sprague-Dawley rats. Cells were cultured in Dulbecco's modified Eagle's medium (DMEM)/nutrient mixture F12 (1:1) containing 10% heat-inactivated fetal bovine serum (Life Technologies, Rockville, MD) and antibiotics (100 U/ml penicillin and 100 μg/ml streptomycin). Culture plates were maintained in the humidified incubator at 37°C with a 5% CO_2 atmosphere. The cells were subcultured after treatment with 0.05% trypsin and 0.53 mM EDTA. Subconfluent cells at passages 4-7 were used for the experiment.

NF-κB decoy oligodeoxyneotides

The sequences of phosphorothioate double-stranded decoy oligodeoxynucleotides against the NF-κB binding site and scrambled oligodeoxynucleotides used in this study were reported previously (Neish *et al.,* 1992; Tomita *et al.,* 1998). Their sequences are as follows: NF-κB decoy oligodeoxynucleotides (5'-CCT TGAAGGGATTTCCCTCC-3'/3'-GGAACTTCCCTA AAGGGAGG-5'), Scrambled oligodeoxynucleotides (5'-TTGCCGTACCTGACTTAGCC-3'/3'-AACGGCATGG ACTGAATCGG-5').

Synthetic oligodeoxynucleotides were washed with 70% ethanol, dried, and dissolved in sterile TRIS-EDTA buffer (10 mmol/l TRIS and 1 mmol/l EDTA). The supernatant was purified on a nucleic acid purification column (NAP-10 Column; Pharmacia LKB Biotechnology, Piscataway, NJ), and the concentrations of oligodeoxynucleotides were quantified by spectrophotometry. The single-stranded oligodeoxynucleotides were annealed for 2 h while the temperature descended from 80 to 25°C.

Transfection of oligodeoxyneotides

Cationic liposomes (Lipofectamine; Life Technologies, Rockville, MD) were used as the transfection vectors for oligodeoxynucleotides into VSMCs. This reagent is a 3:1 (wt:wt) liposome formulation of 2,3-dioleyloxy-N-[2(sperminecarbox-amido) ethyl]-N,N-dimethyl-l-propanaminium trifuoro-acetate (DOSPA) and the neutral lipid dioleoyl phosphatidylethanolamine (DOPE) in membrane-filtered water. Lipid-oligodeoxynucleotides complexes were formed by adding 10 μg lipid in 300 μl OPTI-MEM (Life Technologies, Rockville, MD) to 3.3 μg oligodeoxynucleotides in 300 ml OPTI-MEM. The solution was mixed gently and incubated at room temperature for 45 min to allow liposomes to form. Then 30 μg lipid was used per 75 cm^2 flask to give a final concentration of 2 μmol/l decoy. VSMCs were rendered quiescent by placing them for 24 h prior to transfection in DMEM/F12 medium without fetal bovine serum. Just before transfection, cells were washed with OPTI-MEM medium and then incubated for 3 h at 37°C with freshly prepared liposome-oligodeoxynucleotide complexes. VSMCs were then washed with DMEM/F12 medium and culture was maintained in DMEM/F12 medium without serum at 37°C with 5% CO_2 condition. After confirming that the dose of oligodeoxynucleotides and liposomes used, had no immediate toxic effects on cell viability, fetal bovine serum was added to the culture medium 16 h later. Stable transgene effects were evaluated using fluorescence microscopy to detect fluorescein-labeled oligodeoxynucleotides in transfected cells at 100 h after gene transfection. 5'-fluorescein labeling was mediated by T4 polynucleotide kinase (Promega, Madison, WI).

γ-Irradiation treatment

Forty hours after gene transfection of either 10 μM scrambled or NF-κB decoy oligodeoxynucleotides, cells were exposed to 0, 2, 8, and 16 Gy γ–ray irradiation, using a 137-Cs source delivering 3 Gy/min at room temperature. They were then harvested at 24 and 48 h after radiation by suspension in trypsin and stored at -70°C until assay. NF-κB status after radiation was evaluated by using the rabbit polyclonal antibody to NF-κB p65 (Santa Cruz, Santa Cruz, CA) as primary antibody and FITC-tagged goat anti-rabbit IgG (Biodesign, Saco, ME) as secondary antibody.

Nuclear extraction and NF-κB translocation assay

Samples were harvested 24 h after oligonucleotide transfection and radiation treatment and washed with PBS. Samples were then placed in cold buffer A (10 mM HEPES-KOH (pH7.9), 1.5 mM $MgCl_2$, 10 mM KCl, 0.5 mM DTT, 0.2 mM PMSF) for 10 min. Cell lysates were centrifuged at 1,500 rpm, retreated with cold buffer B (20 mM HEPES-KOH (pH7.9), 25% glycerol, 420 mM Nacl, 1.5 mM $MgCl_2$, 0.2 mM EDTA. 0.5 mM DTT, 0.2 mM PMSF), centrifuged again and the supernatant was extracted.

To measure DNA binding, a NF-κB translocation assay was performed using Mercury TransFactor Kit purchased from BD Biosciences (Palo Alto, CA) following the manufacturer's instructions. Briefly, nuclear extracts were probed on a well coated with *cis*-acting

DNA element for NF-κB binding site and treated with primary and secondary antibodies. After adding substrate and the stop solution 10 min later, the absorbance was measured at 450 nm.

Reverse transcriptase-polymerase chain reaction (RT-PCR)

Primers used for RT-PCR were as follows: TNF-α; F: 5'-CATGATCCGAGATGTGGAACTGGC-3', R: 5'-CTG GCTCAGCCACTCCAGC-3', ICAM-1; F: 5'-CCTTAG GAAGGTGTGATATCCGG-3', R: 5'-AGGTGGTCACC CATGCTGGTGCT-3', iNOS; F: 5'-TCATGATGAAAGA ACTCGGG-3', R: 5'-GAAGGTGAGTTGAACAAGGA-3', GAPDH; F: 5'ACCACAGTCCATGCCATCAC-3', R: 5'-TCCACCACCCTGTTGCTGTA-3'.

Total RNA was isolated using TRIzol reagent (Gibco-BRL, Grand Island, NY). cDNA was acquired by a Reverse Transcription system (Promega, Tokyo, Japan) using 1 μl of total mRNA. PCR reactions contained 1 μM primers, 1.5 mM $MgCl_2$, 200 uM dNTP (deoxynucleotide triphosphate), reaction buffer, 2.5 U Taq DNA polymerase (Promega, Tokyo, Japan), and 1 μl of cDNA in a final volume of 20 μl. PCR conditions were 25 cycles of denaturation at 94°C (45 s), primer annealing at different temperatures for each primer (45 s), extension at 72°C (85 s), and final extension at 72°C (5 min). The PCR products were size fractionated on 1.5% agarose gel.

Western blotting

The following antibodies were used in the present study: anti-TNF-α, anti-NOS2, and anti-ICAM-1 were obtained from Santa Cruz Biotechnology (Santa Cruz, Calif.); and anti-α-tubulin was obtained from Oncogene Research Products (Darmstadt, Germany) Cell lysates were prepared with an ice-cold lysis buffer containing the following: 1 M Tris (pH7.2), 5 M Nacl, NP40, 10% SDS, 0.5 M EDTA, Deoxycholic acid, beta-glyerophosphate, NaF, protease inhibitor, sodium banadate. Equal amounts of protein were electrophoresed on polyacrylamide gels and transferred to polyvinylidene difluoride membranes (Millipore, Billerica, MA). Membranes were blocked with 5% nonfat dry milk (0.1% TBS-T). Positive antibody reactions were visualized with peroxidase-conjugated anti-mouse immunoglobulin G (Amersham Pharmacia, Piscataway, NJ). The peroxidase reaction was performed using an enhanced chemiluminescence detection system (Amersham Pharmacia). The same membrane was reprobed for detection of a different antibody after treatment of the membrane with Restore Western blot stripping buffer (Pierce, Rockford, IL).

Cell proliferation and survival assay

To measure cellular viability and proliferation, the MTT assay using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazoliumbromide was used. Five thousand cells were seeded into a flat-bottomed 96-well-plate and incubated at 37°C in a humidified 5% CO_2

condition. At an appropriate time interval, MTT (2 mg/ml in PBS) was added to the wells. After incubation for 4 h, the resulting color reaction product, MTT formazan, was extracted with dimethyl sulfoxide and the absorbance was measured at 570 nm. Each absorbance value were normalized by the results of NF-κB non-inhibited (scrambled oligonucleotide transfected), non-radiated VSMCs (at 24 h after radiation). The means ± SD (standard deviation) were calculated from the data of 11 sets of microtiter culture plates in each group.

To compare the antiproliferative efficacy of NF-κB decoy oligonucleotides and sulfasalazine, a specific inhibitor of NF-κB, and to examine whether NF-κB decoy has dose-dependent effects, we compared the survival of VSMCs treated with 10 μM scrambled oligonucleotide, 10 μM NF-κB decoy oligonucleotide, 20 μM NF-κB decoy nucleotide, or 100 μM of sulfasalazine, all exposed to 16 Gy radiation. For this analysis, we performed direct cell counting and arbitrarily designated the first measured cell count in the scrambled oligonucleotide group as 100%. All of the other measurements were cell counts relative to the first measurment.

Flow cytometry

VSMCs were cultured in 75-cm[2] flasks, and the irradiated cells were harvested by trypsinization. The percentages of the cells in each cell cycle phase were estimated with the flow cytometry method. Briefly, the cells were washed with PBS (pH 7.4), and then fixed with ice-cold 70% ethanol at 4°C for 4 h. The fixed cells were washed with PBS, and incubated for 30 min at room temperature with 200 μg/ml RNase A in PBS. Then, propidium iodide solution (50 μg/ml) was added. Flow cytometric analysis was performed on FACStar Plus (Becton Dickinson, Franklin Lakes, NJ).

Statistical analysis

Quantified results were expressed as mean ± SD. For non-parametric analysis, Mann-Whitney and Kruskall-Wallis tests were used. Comparison of cell-cycle phase proportions between two groups was performed by χ^2 tests. SPSS 11.0 was used for the statistical analysis, and $P < 0.05$ was considered statistically significant. All experiments were carried out at least 4 times.

Results

NF-κB decoy transfection and inhibition of NF-κB

Radiation induced NF-κB activation and its nuclear translocation was confirmed by immunofluorescence for NF-κB p65 (Figure 1A). DNA binding analysis carried out to confirm the inhibition of NF-κB activation by NF-κB decoy transfection, showed that the increase in DNA binding by NF-κB after irradiation was

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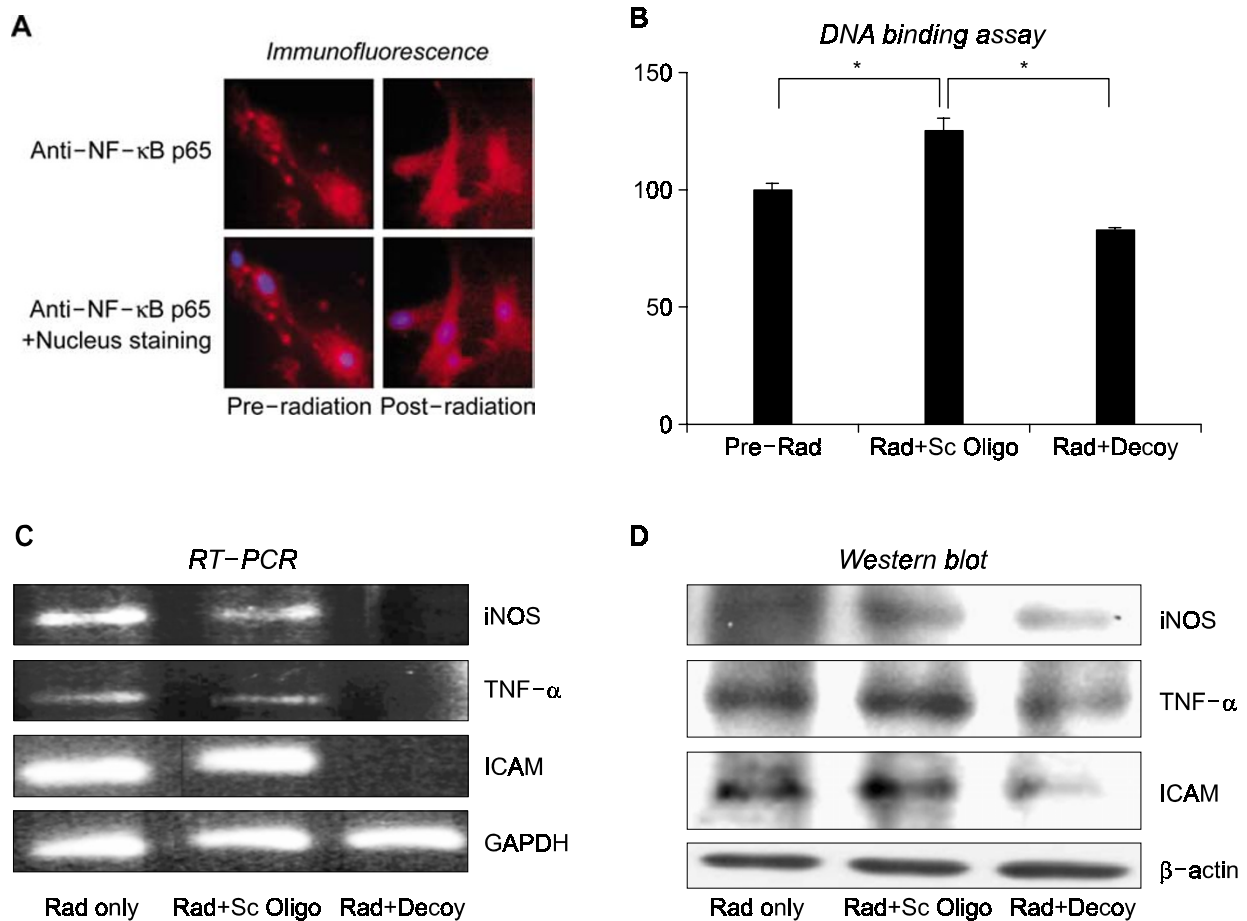
Immunofluorescence

Anti−NF−κB p65

Anti−NF−κB p65
+Nucleus staining

Pre−radiation   Post−radiation

B

DNA binding assay

C

RT−PCR

iNOS

TNF−α

ICAM

GAPDH

Rad only   Rad+Sc Oligo   Rad+Decoy

D

Western blot

iNOS

TNF−α

ICAM

β−actin

Rad only   Rad+Sc Oligo   Rad+Decoy

Figure 1. Activation of NF-κB by radiation and its inhibition by NF-κB decoy transfection. Immunofluorescence staining against NF-κB p65 (red) and merging of the blue stained nucleus showed intranuclear translocation of NF-κB after irradiation (A). DNA binding assay showed inhibition of NF-κB activation by decoy oligodeoxynucleotides. (B). RT-PCR of iNOS, TNF-α, and ICAM, showed that NF-κB decoy transfection reduces transcription of downstream molecules of NF-κB (C), and Western blot analysis showed consequent reduced protein translation (D), confirming inhibition of NF-κB by decoy oligodeoxynuclotides. Rad: 16 Gy radiation (3 Gy/min×320 s), Sc Oligo: 10 μM scrambled oligodeoxynucleotide, Decoy: 10 μM NF-κB decoy oligodeoxynucleotide. *$P < 0.05$.

significantly inhibited by NF-κB decoy transfection (Figure 1B). In addition, RT-PCR and Western blot analysis showed that NF-κB decoy transfection decreased transcription and translation of ICAM, iNOS, and TNF-α, downstream molecules of NF-κB, confirming NF-κB inhibition (Figure 1C and D).

Effects of NF-κB inhibition on irradiated VSMC proliferation and survival

In the non-radiated VSMCs, NF-κB decoy transfection did not show any significant effects on cell survival. However, in the irradiated VSMCs, NF-κB decoy transfection resulted in significant inhibition of VSMC survival and proliferation. At 2 and 8-Gy exposure, radiation only resulted in increased survival of VSMCs compared to non-radiated cells at 24 h after radiation, while exposure to 16-Gy radiation resulted in decreased survival of VSMCs. The transfection of NF-κB decoy in addition to radiation, decreased cellular

survival at all doses of radiation compared with scrambled oligonucleotide transfection, although results were only statistically significant at 8 and 16-Gy (normalized MTT assay: 1.47 ± 0.19 vs. 0.92 ± 0.12, and 0.82 ± 0.24 vs. 0.64 ± 0.09 for 8-Gy and 16-Gy NF-κB non-inhibited vs. inhibited, respectively, $P < 0.01$). At 48 h after radiation, the increase in survival of VSMCs exposed to 2 and 8-Gy radiation and the decrease in survival of cells exposed to 16-Gy radiation compared with non-radiated cells were similar to what was observed at 24 h. However at 48 h, NF-κB decoy transfection significantly inhibited proliferation and survival of VSMCs at all doses of radiation compared with scrambled oligonucleotide transfection (normalized MTT assay: 1.47 ± 0.34 vs. 1.06 ± 0.16, 1.56 ± 0.32 vs. 1.11 ± 0.22, and 1.20 ± 0.25 vs. 0.67 ± 0.19, for 2, 8, and 16-Gy NF-κB non-inhibited vs. inhibited, respectively, $P < 0.01$) (Figure 2A and B). The enhanced inhibition of VSMC survival and proliferation by NF-κB inhibition was such
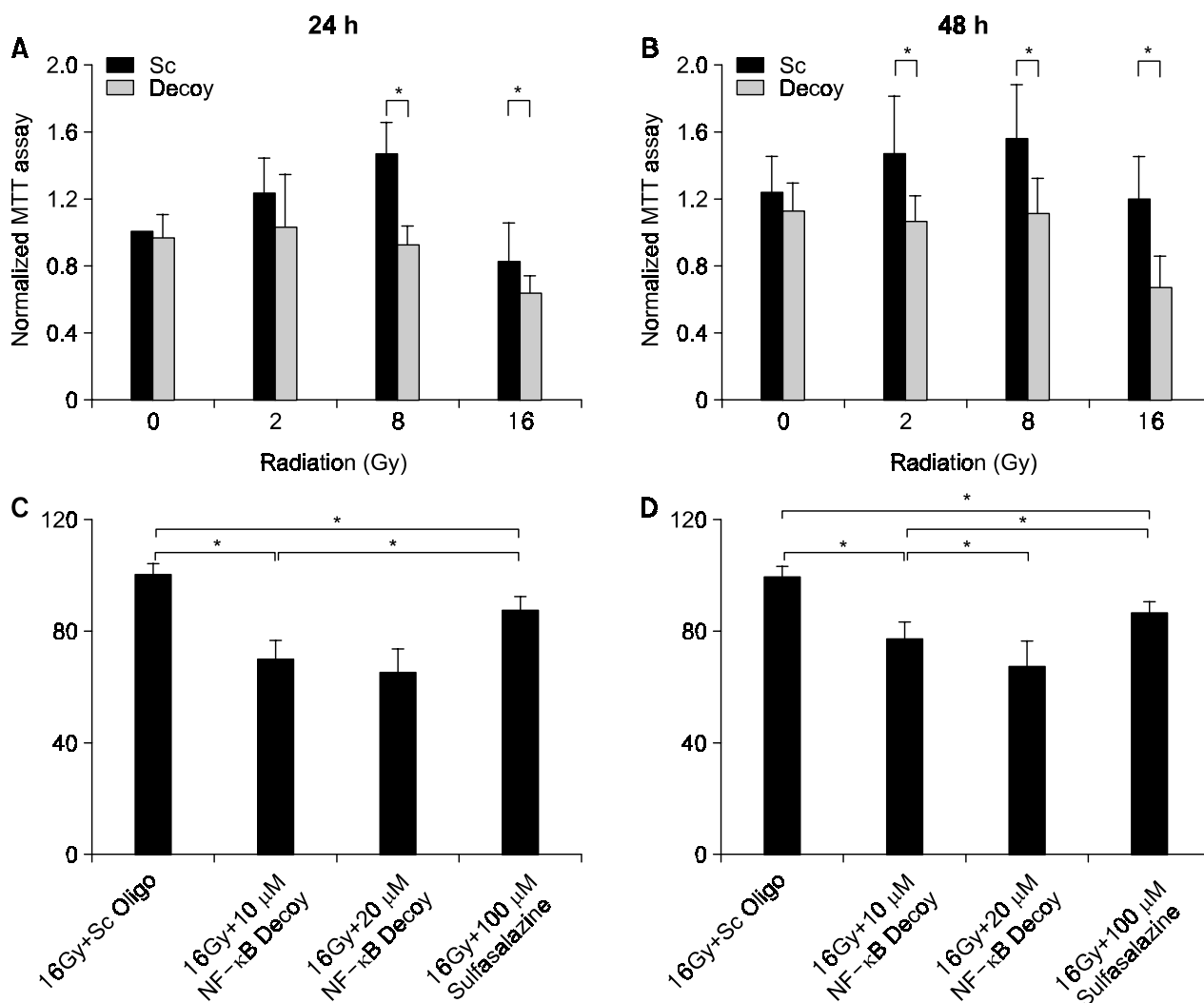
Figure 2. Cell survival assay. 10 μM NF-κB decoy transfection resulted in a significant decrease in survival and inhibition of proliferation in VSMCs exposed to 8 and 16 Gy at 24 h (A), and to all doses of radiation at 48 h (B). Although insignificant at 24 h (C), NF-κB decoy treatment showed a dose responsive effect on cellular survival at 48 h in 16 Gy-irradiated VSMCs. The efficacy of NF-κB decoy was also compared with that of 100 μM sulfasalazine, a specific NF-κB inhibitor, which showed that even the lower dose of NF-κB decoy (10 μM) had a greater inhibitory effect on 16 Gy irradiated VSMCs than 100 μM sulfasalazine at both 24 and 48 h as shown by direct cell counting (C & D). Rad: radiation, Sc Oligo: scrambled oligodeoxynucleotides, Decoy: NF-κB decoy oligodeoxynucleotides. *: $P < 0.05$, NS: not significant. Each absorbance value of MTT assay was normalized by the results of NF-κB non-inhibited non-radiated VSMCs at 24 h.

that at 48 h after radiation, the effects of 2 and 8-Gy radiation combined with NF-κB decoy transfection (1.06 ± 0.16 and 1.11 ± 0.22, respectively) were comparable to that of 16-Gy radiation alone without NF-κB inhibition (1.20 ± 0.25).

In addition, we observed a dose-dependent effect of NF-κB decoy on cell survival. At 48 h in VSMCs exposed to 16 Gy radiation, 20 μM NF-κB decoy transfection showed a further reduction in cell survival from 10 μM NF-κB decoy transfection, (cell survival: $99.9\% \pm 8.8$ vs. $71.1\% \pm 21.3$ vs. $60.7\% \pm 15.4$ for scrambled oligonucleotide vs. 10 μM NF-κB decoy oligonucleotide vs. 20 μM NF-κB decoy oligonucleo-

tide, $n = 4$, $P < 0.05$). Moreover, when the antiproliferative efficacy of NF-κB decoy was compared with that of sulfasalazine, a specific inhibitor of NF-κB, the effects of either 10 and 20 μM NF-κB decoy transfection was greater than that of 100 mM sulfasalazine (cell survival: $99.9\% \pm 8.8$ vs. $71.1\% \pm 21.3$ vs. $60.7\% \pm 15.4$ vs. $85.1\% \pm 15.1$ for scrambled oligonucleotide vs. 10 *m*M NF-κB decoy oligonucleotide vs. 20 μM NF-κB decoy oligonucleotide vs. 100 μM sulfasalazine, $n = 4$, $P < 0.05$).
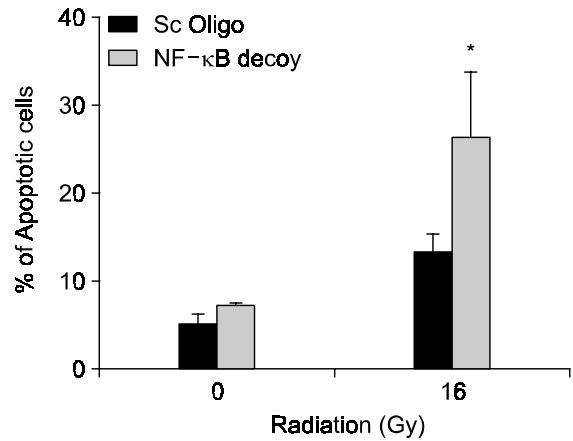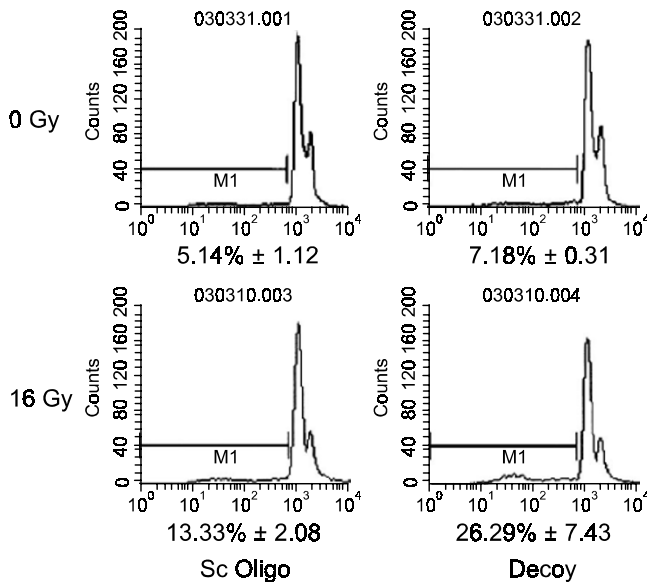
Figure 3. Apoptosis assay. FACS analysis showed that the increase in apoptosis of VSMCs by NF-κB decoy became more significant in 16 Gy-irradiated cells. Sc Oligo: scrambled oligodeoxynucleotides, Decoy: 10 μM NF-κB decoy oligodeoxynucleotides. Proportion of apoptosis cells given as mean ± standard deviation. *: $P < 0.05$ vs. scrambed oligodeoxynucleotide treated VSMCs ($n = 5$).
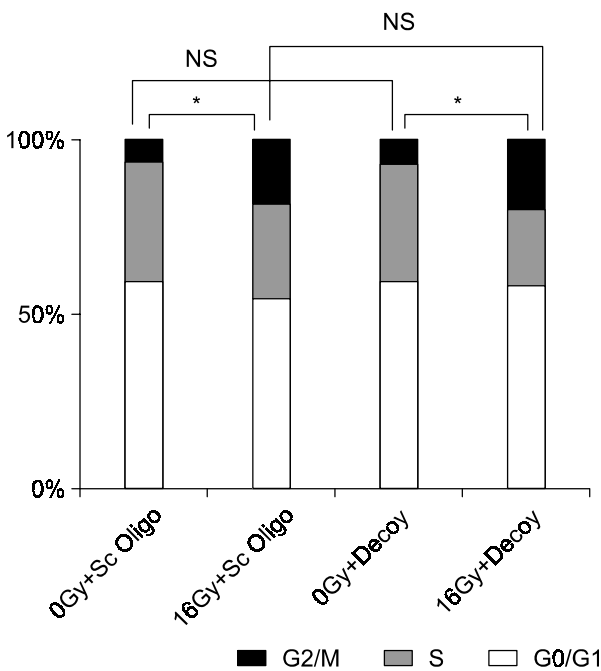

Figure 4. Cell cycle analysis. Radiation exposure resulted in a significant increase in the number of VSMCs in the G2/M phase, and decreased cells in the S-phase, a profile of G2 arrest as measured by FACS analysis of the cell cycle. However, compared with scrambled oligodeoxynucleotide treatment, transfection of NF-κB decoy oligodeoxynucleotide did not result in any significant additive effects on the cell cycle. Sc Oligo: scrambled oligodeoxynucleotides, Decoy: 10 μM NF-κB decoy oligodeoxynucleotides. *: $P < 0.05$, NS: not significant.

Effects of NF-κB inhibition on irradiated VSMC apoptosis

FACS analysis showed that the proportion of apoptotic cells significantly increased after radiation treatment (apoptosis fraction: 5.14 ± 1.12% vs. 13.33 ± 2.08%, $P < 0.05$). Furthermore, the apoptotic fraction of irradiated VSMCs was significantly increased by NF-κB decoy transfection (13.33 ± 2.08% vs. 26.29 ± 7.43%, $P < 0.05$) showing that NF-κB inhibition significantly enhanced the proapoptotic effects of radiation (Figure 3).

Effects of NF-κB inhibition on the cell cycle of irradiated VSMCs

γ-ray irradiation itself induced cell cycle arrest, reducing the percentage of cells in the S-phase and increasing the percentage of cells in the G2/M phase, a typical profile of G2 arrest (G0/G1:S:G2/M = 59%: 35%:6% vs. 54%:28%:18%, non-radiated vs. 16-Gy radiated cells at 24 h after radiation, $P < 0.01$). Further inhibition of NF-κB in irradiated VSMCs, did not show any additional effects in terms of cell cycle arrest (G0/G1:S:G2/M = 54%:28%:18% vs. 58%:22%: 20%, NF-κB non-inhibited vs. inhibited in 16-Gy radiated cells at 24 h, $P = 0.348$) (Figure 4).

Discussion

NF-κB, a cell survival factor activated by radiation in VSMCs, may render such cells to be relatively radioresistant. Such radioresistence requires us to use high doses of local radiation to inhibit VSMC proli-

feration and neointimal growth after angioplasty in clinical practice during coronary brachytherapy. Therefore, we studied whether inhibition of NF-κB through NF-κB decoy transfection would potentiate the antiproliferative effects of radiation. We showed that NF-κB decoy transfection inhibits the activation of NF-κB and its subsequent transcription and translation of downstream molecules. We also found that such inhibition of NF-κB leads to decreased survival and proliferation of irradiated VSMCs, which seem to be due to increased apoptosis of VSMCs rather than additional cell cycle arrest.

It is well-known that ionizing radiation including γ-rays activates NF-κB (Li and Karin, 1998). We postulated that inhibition of NF-κB activation would reduce survival and inhibit proliferation of irradiated VSMCs. In the present study, NF-κB inhibition significantly reduced survival and proliferation of VSMCs exposed to 8 and 16-Gy radiation at 24 h and all doses of radiation at 48 h. However NF-κB decoy transfection had no significant effects on non-radiated VSMCs. Due to the fact that NF-κB in resting cells is sequestered in the cytoplasm bound to IκB$_α$ and IκB$_β$ (Baldwin, 1996), we believe that NF-κB inhibition by decoy oligodeoxynucleotides has little effect under conditions where NF-κB is not activated, such as non-radiated VSMCs in serum-rich culture conditions.

In addition, the efficacy of 10 μM NF-κB decoy, at both 24 and 48 h, was greater than that of 100 μM sulfasalazine, a specific inhibitor of NF-κB. Furthermore, the effect of 20 μM NF-κB decoy was greater than that of 10 μM NF-κB decoy at 48 h, suggesting a dose dependent effect of NF-κB decoy.

NF-κB has been shown in various cells to have an anti-apoptotic effect. Impaired NF-κB has been associated with massive apoptosis during development in several gene knockout studies (Li *et al.,* 1999; Tanaka *et al.,* 1999). In addition, NF-κB has been shown to protect neurons from beta-amyloid peptide induced apoptosis in Alzheimer's disease (Bales *et al.,* 1998; Guo *et al.,* 1998). Furthermore, NF-κB inhibition using decoy oligonucleotide was associated with enhanced apoptosis in neuronal cells (Guo *et al.,* 1998), and with TNF-α induced apoptosis in prostate cancer cell lines (Sumitomo *et al.,* 1999). In the present study, the simple inhibition of NF-κB, in non-radiated VSMCs, showed only minimally increased apoptosis, which were not statistically significant (5.14 ± 1.12% vs. 7.18 ± 0.31%, for NF-κB non-inhibited vs. inhibited, $P > 0.05$). However, in irradiated VSMCs, NF-κB inhibition resulted in a significantly increased apoptotic cell fraction (13.33 ± 2.08% vs. 26.29 ± 7.43%, for NF-κB non-inhibited vs. inhibited, $P < 0.05$).

Ionizing radiation is known to induce cell cycle arrest (Brugarolas *et al.,* 1995; Scott *et al.,* 2001). Radiation activates p53 *via* ATM, which subsequently, activates p21^{Waf-1}, a cdk inhibitor that induces G1 and G2 arrest by cdk/cyclin inhibition (Jonathan *et al.,* 1999). In contrast to the established data on the role of radiation, relatively little is known regarding the role of NF-κB in the progression of the cell cycle. There

has been a conflicting evidence on whether NF-κB is important in the progression or the arrest of the cell cycle. Some have reported that NF-κB activation is necessary to cause cell cycle arrest (Bash *et al.,* 1997). However the body of evidence in recent studies have shown that NF-κB activation is related to cell cycle progression rather than arrest in various cell types (Baldwin *et al.,* 1991; Cressman *et al.,* 1994; Yamada *et al.,* 1997), and that NF-κB inhibition results in cell cycle arrest (Kaltschmidt *et al.,* 1999; Otsuka *et al.,* 1999). Radiation has bi-directional effects on p53; it can activate p53 *via* ATM (Herzog *et al.,* 1998; Jonathan *et al.,* 1999) and simultaneously activates NF-κB (Li and Karin, 1998), which may have an inhibitory effect on p53 (Stoffel *et al.,* 2004). Therefore the balance of p53 activation and inhibition may determine the fates of the irradiated cells. In the present study, radiation significantly reduced the fraction of cells in the S-phase and increased cells in the G2/M phase, a typical profile of G2 phase arrest. However, we could not observe any additive effects of NF-κB inhibition on the irradiated cells. NF-κB decoy treatment in the setting of the present study (dose and culture conditions) may not have altered p53 balance enough to cause a significant change in the cell cycle.

Many clinical studies of intracoronary radiation therapy have reported successful reduction of restenosis (Condado *et al.,* 1997; Teirstein *et al.,* 1997; King *et al.,* 1998; Waksman *et al.,* 2000). The main mechanism of radiation is to inhibit the proliferative response of VSMCs at the site of injury (Waksman *et al.,* 1997). Due to the high doses of radiation, adverse effects such as aneurysmal dilatation of the artery and late thrombosis have been detected in clinical studies (Condado *et al.,* 1999; Farb *et al.,* 2003). These adverse effects may be decreased by low-dose radiation. As the present study shows, low-dose radiation combined with NF-κB inhibition may be comparable to the effects of high-dose radiation in regard to VSMC proliferation and survival. Furthermore, the dose dependent effects and efficacy of NF-κB decoy shown in the present study, suggests that it may be a simple and efficacious modality of NF-κB inhibition. It was recently shown that NF-κB decoy reduces neointima in a porcine model of coronary artery injury through inhibition of proinflammatory genes and adhesion molecules (Yamasaki *et al.,* 2003). Furthermore, the authors hypothesized that one of the mechanisms of neointima inhibition, may be the inhibition of injury-activated VSMC proliferation by induction of VSMC apoptosis (Morishita *et al.,* 2004). However, this was not a model of intracoronary radiation but only simple vessel injury. We believe that the effects of NF-κB decoy would be more efficacious in the setting of intracoronary radiation along with vessel injury, which is what goes on after coronary brachytheray.

In conclusion, we found that NF-κB inhibition potentiates the antiproliferative effects of radiation in VSMCs and these effects seem to be through increased apoptosis of VSMCs rather than additional

cell cycle arrest. Although our *in vitro* data must be confirmed first in animal models of restenosis and VSMC hyperplasia, the results of the present study suggest the possibility of combined intracoronary radiation with NF-κB inhibiting gene therapy, so called "radiogene therapy", to inhibit VSMC proliferation and neointimal growth after angioplasty.

Acknowledgement

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