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Ionizing Radiation Induces, via Generation of Reactive Oxygen Intermediates, Intercellular Adhesion Molecule-1 (ICAM-1) Gene Transcription and NFκB-like Binding Activity in the ICAM-1 Transcriptional Regulatory Region

HERMANN BAEUML[a], UTA BEHRENDS[b], RALPH U. PETER[a], STEFAN MUELLER[a], CLAUDIA KAMMERBAUER[a], S. WRIGHT CAUGHMAN[c] and KLAUS DEGITZ[a,*]

[a]*Department of Dermatology, Ludwig-Maximilians-University, Frauenlobstr. 9-11, München, Germany;* [b]*Institute of Molecular Virology, GSF, Neuherberg, Germany;* [c]*Department of Dermatology, Emory University School of Medicine, Atlanta, GA, USA*

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Ionizing radiation produces reactive oxygen intermediates in mammalian tissues and may serve as a model system for the investigation of the biologic effects of free radicals. We have previously shown that the adhesion molecule ICAM-1 is induced by ionizing radiation, and here we have investigated the molecular mechanisms responsible. ICAM-1 mRNA and cell surface expression was induced in HeLa and HaCaT cells after exposure to ionizing radiation. This induction was blocked by preincubation with the antioxidants PDTC and N-acetyl cysteine. ICAM-1 promoter activity was assessed by transiently transfecting HeLa cells with CAT-reporter gene constructs containing sequential ICAM-1 5′ deletions. ICAM-1 5′ fragments –1162/+1 (relative to the transcription start site) and –277/+1 displayed increased promoter activity when cells were exposed to ionizing radiation, but no induction was seen in a –182/+1 construct associating positions –277 to around –182 with inducibility by ionizing radiation. Nuclear extracts from HaCaT cells were tested in mobility shift assays using an NFκB-like binding site of the ICAM-1 5′ region (positions –186/–177). There was marked enhancement of DNA-protein complex forming in extracts from irradiated *versus* untreated cells. Incubation of cells with antioxidants prior to irradiation prevented the radiation-dependent increase in complex formation. We conclude that reactive oxygen intermediates are involved in ICAM-1 induction by ionizing radiation. The ionizing radiation-induced, antioxidant-inhibitable binding at the ICAM-1 NFκB-like binding site is consistent with the view that NFκB is a pro-oxidant transcription factor.

Keywords: Gene regulation, adhesion molecules, inflammation, epithelial cells

INTRODUCTION

Intercellular-adhesion molecule 1 (ICAM-1) is one of several cell surface molecules involved in adhesion events among leukocytes and between

* Corresponding author. Tel.: 49-89-51604149. Fax: 49-89-51604551.

leukocytes and other cell types. Binding of ICAM-1 to its ligands, the leukocyte intergrins LFA-1 (CD11a/CD18) or Mac-1 (CD11b/CD18), has been implicated in leukocyte extravasation, target cell lysis by cytotoxic T cells, and antigen presentation to T cells.[1] Additionally, ICAM-1 may be involved in cell binding to the extracellular matrix, since it also binds hyaluronic acid.[2] ICAM-1 is a glycosylated transmembrane protein with a molecular mass ranging from 76 to 114 kDa, depending on tissue-specific glycosylation.[3] It is a member of the immunoglobulin superfamily and possesses five extracellular immunoglobulin-like domains.[4] ICAM-1 is constitutively expressed on vascular endothelial cells and, in low amounts, on a few other tissues including hematopoietic cells, fibroblasts, and certain epithelial cells.[5] It can be upregulated on these cell types and induced *de novo* on many others by proinflammatory cytokines including TNF-α, IL-1 and IFN-γ,[5,6] but also by non-physiologic proinflammatory stimuli like bacteial cell wall components (LPS), phorbol esters,[7] and electromagnetic waves including UV-A and UV-B,[8–10] and, as we have recently shown, by ionizing radiation (IR).[11]

The induction of surface expression is the principal measure for the regulation of ICAM-1 function in the initiation and maintenance of inflammatory diseases, and the molecular mechanisms underlying ICAM-1 induction have thus been intensively studied. ICAM-1 expression is primarily regulated at the transcriptional level, although posttranscriptional regulatory mechanisms may also be effective.[12] The transcriptionally regulatory regions of the human[13–17] and mouse[18] ICAM-1 gene have been structurally characterized. Studies combining analyses of promoter function and transcription factor binding patterns have led to the identification of several principal transcriptional activation mechanisms. A nuclear factor κB (NFκB) site at positions –187/–178 relative to the main transcription start site was demonstrated to confer transcriptional activation elicited by phorbol esters and TNF-α;[17,19–21] an IFN-γ responsive element was located at positions –76/–66;[20,22,23] and a retinoic acid response element was identified at –226/–215.[24]

IR produces reactive oxygen intermediates in mammalian tissues[25] and may serve as a model system for the investigation of the biologic effects of free radicals. We have previously shown that ICAM-1 is induced by IR at the mRNA and cell surface levels.[11] The rapid induction of ICAM-1 occurred at the level of transcription, was independent of de novo protein synthesis, and did not involve autocrine stimulation via TNF-α and IL-1.

Here we have investigated the molecular mechanisms of ICAM-1 induction by IR. We demonstrate that ICAM-1 induction by IR is mediated by the same NFκB site that also mediates induction by other pro-oxidant stimuli, such as PMA and TNF-α. Antioxidants inhibited both ICAM-1 expression and activation of binding at the NFκB-like binding site.

MATERIALS AND METHODS

Cell Culture and Reagents

HaCaT, a spontaneously immortalized, nontumorigenic keratinocyte cell line,[26] was a kind gift from N. Fusenig, DKFZ, Heidelberg, Germany. The human cervical carcinoma cell line HeLa was obtained from American Type Culture Collection, Rockville, MD. Cells were cultured in DMEM supplemented with 2 mM L-glutamine, antibiotics (100 U/ml penicillin and 100 μg/ml streptomycin) and 1 μg/ml amphotericin B (all from Gibco/BRL, Eggenstein, Germany), and 10% fetal calf serum (Biochrom, Berlin, Germany), at 37°C and 5% CO2. In some experiments, pyrrolidine dithiocarbamate (PDTC) or N-acetyl cysteine (both Sigma, Diesenhofen, Germany) were added to cultures at varying con-

centrations. In the used concentrations, PDTC and N-acetyl cysteine did not affect cell viability during the time period relevant for each particular experiment.

Irradiation

Ionizing radiation was delivered to cells as previously described.[11] Briefly, cell cultures were exposed to single doses of IR delivered by bilateral irradiation from a 137Cs source (Gammacell, HWM-D-2000, Ottawa, Canada) at a dose rate of 0.95 Gy/min. Unirradiated controls were removed from the incubator during the time required for the irradiation procedure.

Northern Blot Analysis

Total cellular RNA was isolated from cells as described by guanidinium isothiocyanate lysis, CsCl density gradient pelleting, formaldehyde/agarose gel electrophoresis, and blotting to nylon membranes.[27] A 1,2 kb Sal I-Kpn I-fragment of the ICAM-1 cDNA clone pGH1.1ms (kindly provided by D. Staunton, Dana-Faber Cancer Institute, Boston, MA) was radiolabeled with [α-^{32}P] dCTP (Hartmann, Braunschweig, Germany) via random hexamer primer extension and used as a hybridization probe. As a control for loading uniformity, a 0.21 kb PCR product amplified from a human glyceraldehyde-3-phosphate dehydrogenase cDNA was used as previously described.[11] Prehybridization and hybridization were carried out at 42°C in 50% formamide, 1M NaCl, 10% dextran, 1% SDS, 100 µg/ml yeast tRNA and 10 mg/ml salmon sperm DNA. After overnight hybridization, membranes were washed under increasingly stringent conditions and exposed to XAR-5 films with one intensifying screen at –70°C over night. Quantitative analysis was carried out using a CS-1 scanner (Cybertech, Berlin, Germany) and WinCam software (Cybertech).

Immunofluorescence Flow Cytometry

Cell surface expression of ICAM-1 was assessed by a one step staining procedure and subsequent immunofluorescence flow cytometry (FACS analysis). Untreated or irradiated cells were incubated with a FITC-coupled murine anti-human ICAM-1 mAb (84H10, Bender Med Systems, Vienna, Austria) or with an FITC-coupled isotype-matched control mAb (mouse IgG1, Dianova, Hamburg, Germany). Subsequently, cells were analyzed in a FACScan II flow cyto-meter using the Lysis II analysis program (Becton Dickinson, Heidelberg, Germany) as previously described.[11] Briefly, ICAM-1 surface expression was quantified as mean fluorescence intensity of staining with the anti ICAM-1 mAb and was corrected for autofluorescence by substracting the mean fluorescence intensity value obtained with the isotype control mAb.

Assessment of Cell Viability

Cell viablility was assessed at varying time points after exposure to IR by flow cytometry as described previously.[11] Briefly, 50 ng propidium iodide (Sigma) were added to cells and FACS analysis performed for the distinction of intact (propidium iodide negative) from dead (propidium iodide positive) cells.

ICAM-1-based Chloramphenicol Acetyl Transferase (CAT) Reporter Gene Constructs

Chloramphenicol acetyl transferase (CAT)-expression vectors containing various portions of the ICAM-1 5′ flanking region have been described previously.[14,21] Briefly, purified ICAM-1 5′ fragments were cloned into a modified parent CAT plasmid, pCAT-Promoter (Promega, Madison, WI), from which the SV40 promoter had been removed via a BgI II/Stu I digestion. Inserts and ligation sites of all vectors were confirmed by sequence analysis via the dideoxynucleotide chain

termination method using the Sequenase 2.0 reaction kit (U.S. Bio-chemical, Cleveland, OH). All fragments are designated by their most 5′ nucleotide position relative to the identified transcription initiation site located 40 bp 5′ of the translation start codon.[14] Schematics of the ICAM-1-based CAT constructs utilized are shown in Figure 1.

Transient Expression of CAT Vectors

Subconfluent HeLa cell cultures (1.5×10^6 cells/100 mm tissue culture dish) were transfected by the calcium phosphate precipitation technique with 20 µg of plasmid DNA of various ICAM-1 constructs as described previously[14,21] with minor modifications. Briefly, transfection experiments with pCAT-Basic (Promega), which is a vector without any endogenous promoter or enhancer, served as a negative control for CAT expression. 10 µg of a beta-galactosidase expression vector (pSV-Beta-Galactosidase Control Vector, Promega) were cotransfected along with the various ICAM-1-CAT-expression vectors as a control for transfection efficiency among various plates and constructs. After exposure to precipitated plasmids for 16 h , cells were washed and replenished with medium. Transfected cells were either left untreated or irradiated with 20 Gy of IR 18 h prior to lysis. Cell lysates were prepared 48 h after transfection as described.[14] Lysates were assayed for beta-galactosidase activity using the Beta-Galactosidase Enzyme Assay System (Promega). Lysates were subsequently used for CAT-Assays in volumes normalized for beta-galactosidase activity. CAT enzyme activity of normalized lysates was determined using ^{14}C-labeled chloramphenicol (New England Nuclear, Bad Homburg, Germany). Acetylated and non-acetylated forms of chloramphenicol were resolved by thin layer chromatography and visualized by autoradiography (exposure to a Kodak XAR-5 film at room temperature over night). The percentage of acetylated chloramphenicol was determined by scintillation counting of appropriate areas of the chromatogram.

Preparation of Nuclear Extracts

Nuclear extracts were prepared as previously described.[28] Cells were kept in serum-free medium 16 h prior to lysis. Nuclei were isolated either from control HaCaT cells or from HaCaT cells stimulated with 20 Gy of IR for 1 h. Protein

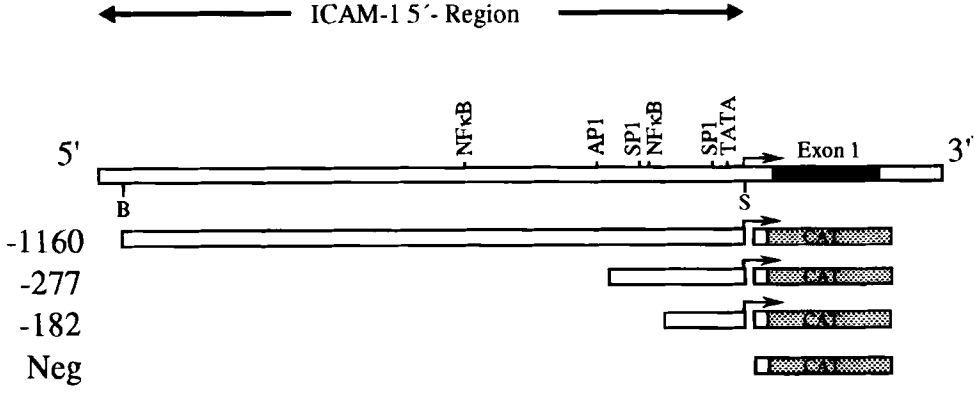

FIGURE 1 **Schematic depiction of a 2.05-kB ICAM-1 gene fragment[14] containing the immediate 5′ flanking region, and ICAM-1-based CAT reporter gene constructs**. The genomic subfragment contains 1350 bp of 5′ flanking region, the transcription start site (marked by a bent arrow), the first exon (black area), and part of the first intron. The relative locations of transcription factor binding sequences (NFκB, AP1, SP1, TATA-Box) and restriction endonuclease sites (B, Bgl II; S, Sst I) are indicated. Restriction fragments or PCR-generated deletion mutants were cloned into CAT expression vectors and are designated according to the most 5′ base position incorporated.

concentrations of extracts were determined photometrically using the Coomassie protein assay reagent (Pierce, Rochford, Ill) and were normalized for protein concentration prior to assay.

Electrophoretic Mobility Shift Assays

15 µg of nuclear extract were incubated with ^{32}P-radiolabeled double stranded oligonucleotide probes in a 20 µl reaction mix containing 0.225 mg/ml bovine serum albumin (Sigma), 0.2 mg/ml poly dI/dC (Pharmacia, Uppsala, Sweden), 12 mM HEPES, 4 mM Tris (pH 7.9), 100 mM KCl, 1 mM EDTA, 1 mM DTT, 12.5% (w/v) glycerol. Single stranded deoxyoligonucleotides representing ICAM-1 genomic DNA were synthesized by MWG Biotech, Ebersberg, Germany. Additionally, consensus binding sequences for transcription factors were commercially obtained (Promega). Annealed double stranded oligonucleotides with 5′ overhangs were radiolabeled with [α-^{32}P] dATP (Hartmann) using the Klenow fragment of DNA polymerase I (AGS, Heidelberg, Germany). Annealed double stranded oligonucleotides with blunt ends were radiolabeled with [γ-^{32}P] ATP (Harmann) using T4 polynucleotide kinase (Boehringer Mannheim, Mannheim, Germany). The oligonucleotide probes used in this study are summarized in Table I. DNA/protein binding complexes were resolved by electrophoresis on non-denaturing 5% polyacrylamide gels in a high ionic strength glycine (14.3% w/v) running buffer. Gels were run at 12 V/cm for 2 h. Dried gels were exposed to Kodak XAR-5 film at –70°C with one intensifying screen.

For supershifts of DNA-protein binding complexes, antisera directed against transcription factors were added to nuclear extracts at a concentration of 300 ng/µl 2 h prior to the addition of labeled DNA probes. Polyclonal antisera against subunits of the NFkB family of transcription factors were obtained from Santa Cruz Biotechnology, Santa Cruz, CA. These antisera were specific for p50, p65, c-Rel, RelB, and p52/ NFκB2. Furthermore, a polyclonal antiserum against the transcription factor c-Fos was utilized.

Statistical Analysis

Values are expressed as mean ± standard deviation. The Mann-Whitney U test was used to evaluate statistical differences of test and control groups, when irradiated and test groups were compared to untreated controls.

TABLE I Double stranded oligonucleotides used in Electrophoretic mobility shift assays

Sequence of double stranded oligonucleotides[a]	Position in ICAM-1 5′-region[b]	Type of Binding site	Binding motif	Position of motif in ICAM-1 5′-region[b]
5′GATCTAGA**GGAGGGGATCCCTCA**TCTA 3′ ATCT**CCTCCCCGTAGGGAGT**AGATCTAG	–531/–516	NFκB	5′GGGGCATCCC	–528/–519
5′**ATTGCTTTAGCTTGGAAATTCCGGAGCTGA** 3′**TAACGAAATCGAACCTTTAAGGCCTCGACT**	–199/–170	NFκB	5′CGGAATTTC	–186/–177[c]
5′AGTTGAGGGGACTTTCCCAGGC 3′TCAACTCCCCTGAAAGGGTCCG		NFκB consensus	5′GGGACTTTCC	
5′CGCTTGATGAGTCAGCCGGAA 3′GCGAACTACTCAGTCGGCCTT		AP-1 consensus	5′TGAGTCA	
5′ATTCGATCGGGGCGGGGCGAGC 3′TAAGCTAGCCCCGCCCCGCTCG		SP-1 consensus	5′GGGCGG	

[a] Bold letters represent genomic ICAM-1 sequence.
[b] Relative to the transcription start site,
[c] Actual sequence of binding motif is contained in antisense strand.

RESULTS

ICAM-1 mRNA and Cell Surface Expression is Upregulated by IR, and this Upregulation is Inhibited by Preincubation with Antioxidants

Irradiation with 40 Gy of IR increased ICAM-1 mRNA expression in HaCaT (Fig. 2) and HeLa cells (not shown) 6 h post-irradiation, which is the time point around which ICAM-1 mRNA induction by IR peaks.[11] ICAM-1 mRNA was increased 2.9-fold in HaCaT and 3.5-fold in HeLa cells in IR-treated cells compared to controls. ICAM-1 mRNA induction was blocked when cells were incubated with the antioxidant PDTC (100 μM) 30 min prior to irradiation both in HaCaT (Fig. 2) and HeLa cells (data not shown). Preincubation with the antioxidant N-acetyl cysteine (100 nM) also markedly inhibited ICAM-1 mRNA induction by IR in HeLa cells (data not shown). No loss of viability compared to unirradiated controls was seen in HaCaT and HeLa cells 6 h after exposure to 40 Gy of IR.

ICAM-1 surface protein was assessed 24 h after exposure to IR. The IR-dose had to be reduced to 20 Gy, because higher doses markedly affected cell viability 24 h post-irradiation. No loss of cell viability was seen in HeLa and HaCaT cells 24 h after exposure to 20 Gy compared to unirradiated controls as determined by propidium iodine uptake. IR increased ICAM-1 surface expression in HeLa cells as assessed by immunofluorescence flow cytometry (Fig. 3). The mean fluorescence intensity was 9.9 ± 2.1 ($n = 7$) in unirradiated cells and was increased to 17.6 ± 2.2 ($n = 7$) post-irradiation. This increase of ICAM-1 cell surface expression was statistically significant ($p = 0.017$). Incubation with N-acetyl cysteine reduced ICAM-1 surface expression both in unirradiated (6.5 ± 1.21; $n = 7$) and irradiated cells (11.3 ± 1.0; $n = 7$). The reduction of ICAM-1 surface expression by N-acetyl cysteine was significant both for unirradiated ($p = 0.018$) and irradiated ($p = 0.017$) cells. IR also increased ICAM-1 surface expression in HaCaT cells (data not shown) as we have previously described.[11]

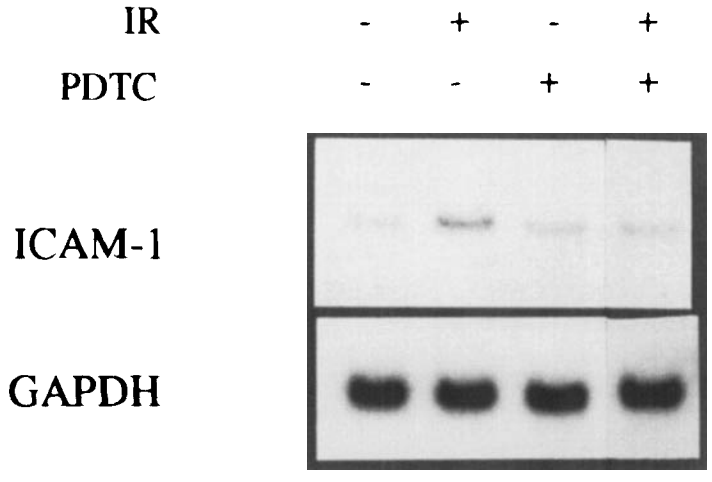

FIGURE 2 **Ionizing radiation induces ICAM-1 mRNA in HaCaT cells, and this induction is blocked by PDTC.** Total cellular RNA was isolated from untreated controls and from irradiated cells 6 h after IR (40 Gy). Additionally, unirradiated control cells as well as irradiated cells were incubated with 100 μM PDTC 30 minutes prior to irradiation. RNA was electrophoresed and analysed by Northern-blot hybridization using a ^{32}P-labeled human ICAM-1 cDNA probe. A PCR product amplified from a human glyceraldehyde-3-phosphate dehydrogenase cDNA is shown as a control for loading uniformity.

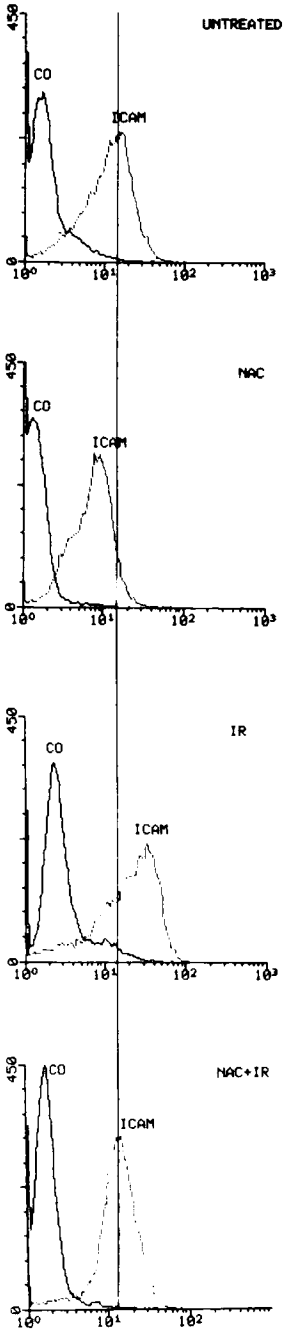

FIGURE 3 **Ionizing radiation induces ICAM-1 cell surface expression in HeLa cells**. Analysis of untreated controls and irradiated cells 24 h after exposure to IR. Additionally, unirradiated control cells as well as irradiated cells were incubated with 50 mM N-acetyl cysteine 30 minutes prior to irradiation. Immunofluorescence flow cytometry was performed with either IgG1 isotype control monclonal antibody (CO) or anti-ICAM-1 monclonal antibody (ICAM).

An IR-responsive Region is Located in the 5′ Region of the Human ICAM-1 Gene

The induction of ICAM-1 mRNA by IR is predominantly transcriptionally regulated.[11] It was, therefore, attempted to identify gene segments which mediate ICAM-1 transcriptional activation in response to IR. HeLa cells were transiently transfected with CAT-reporter gene constructs containing sequential 5′ deletions of the 5′ region of ICAM-1 (Fig. 1). Transfected cells were either left untreated or irradiated with 20 Gy of IR. ICAM-1 promoter activity was subsequently assessed in CAT assays (Fig. 4). Reporter constructs containing fragments –1160/+1 and –277/+1 displayed an increase in promoter activity when cells were exposed to IR. However, no induction was observed in ICAM-1-CAT construct –182/+1, nor with the promoterless expression vector pCAT Basic, indicating that ICAM-1 sequences between positions –277 and around –182 were most relevant for the ICAM-1 inducibility by IR. Induction of the ICAM-1-CAT constructs by IR was also observed in transiently transfected HaCaT cells (data not shown).

IR Induces NFκB-like Binding Activity in the 5′ Region of the Human ICAM-1 Gene, and this Induction can be Inhibited by Antioxidants

At the 3′ end of the segment most relevant for IR-induction in the promoter studies, there lies an NFκB-like binding site (5′ CGGAATTTCC 3′, positions –186/–177) that we and others have previously demonstrated to be crucial for induction of ICAM-1 transcription by phorbol esters[19,21] and TNF-α.[17,20] The construction of the ICAM-1 CAT-expression vector –182/+1 disrupted the IR-inducible NFκB-like binding motif at positions –186/–177, and this truncation coincides with an abrupt reduction of IR-inducibility of the –182/+1 CAT-construct compared to constructs containing larger fragments (Fig. 4). We therefore tested whether this NFκB-

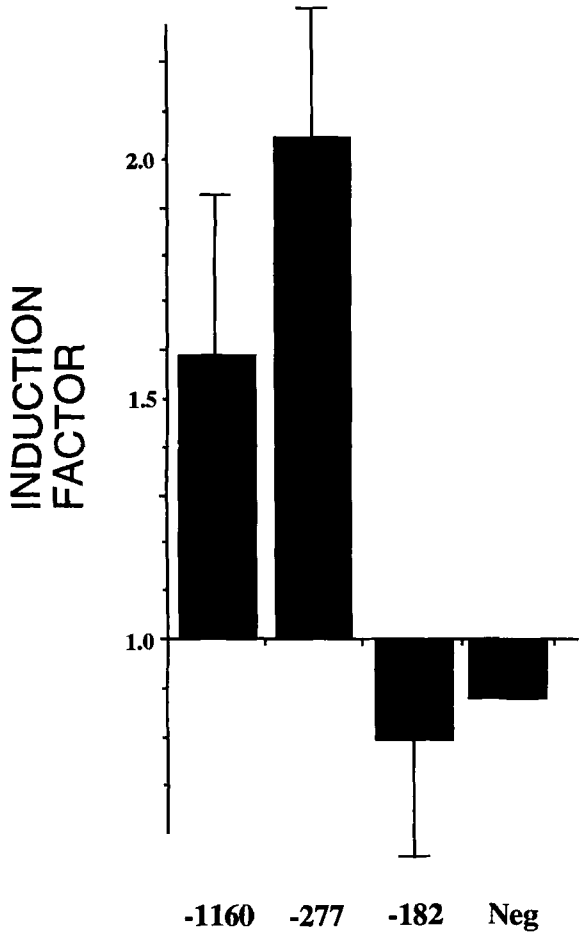

FIGURE 4 **Ionizing radiation activates ICAM-1 based CAT reporter gene constructs**. Various ICAM-1 based CAT plasmids were transiently transfected into HeLa cells, exposed to 20 Gy of IR, and cell lysates prepared 18 h after irradiation. Promoter inducibility is expressed as the ratio of CAT activity of lysates from irradiated transfectants divided by the CAT-activity of lysates from untreated transfectants. Inducibility ratio (Induction factor) is depicted as mean ± SD from 3 independent experiments. Neg, ratio for cells transfected with the promoterless CAT-construct pCAT Basic (one representative experiment).

like binding site was directly involved in IR-induction of ICAM-1 transcription. A double stranded oligonucleotide probe including the NFκB-like binding site (Table I) was investigated in electrophoretic mobility shift assays against nuclear extracts prepared from untreated or IR-irradiated HaCaT cells. While baseline DNA-protein complexes were formed when extracts from untreated cells were used, there was a marked enhancement of complex formation in extracts from irradiated (20 Gy) *versus* untreated cells (Fig. 5). Incubation of cells with PDTC (100 μM) 1 h prior to IR prevented binding activation (Fig. 5). Similarly, when a double stranded oligonucleotide probe containing an NFκB consensus binding site (identical to the NFκB sites of HIV-1; Table I) was used as a probe, an increased DNA-protein complex formation was observed in irradiated compared to untreated nuclear extracts (Fig. 5) confirming previous observations.[29] The nuclear extracts were also reacted with a double-stranded oligonucleotide containing a consensus sequence for SP1, a constitutively expressed transcription factor not influenced by a wide range of stimuli. The SP1 binding activity was unchanged among extracts, making it unlikely that the observed differences in binding activity reflect differences in extract composition or preparation. Two inducible binding complexes were found to be sequence specific for NFκB (Fig. 6), because they were competed away by excess unlabeled identical double stranded oligonucleotide and also by a double-stranded oligonucleotide with an NFκB consensus binding motif. However, the binding was unaffected by the presence of a double-stranded oligonucleotide of irrelevant sequence (AP1 consensus motif, Table I).

p65/RelA and p50 are Constituents of the IR-induced Binding Complex at the NFκB-like Binding Site at Positions –186/–177

Nuclear extracts from HaCaT cells treated with 20 Gy of IR were exposed to antisera directed against individual members of the NFκB family of transcription factors (Fig. 7). When compared to a reaction without added antiserum, a reaction preincubated with an antiserum against an irrelevant protein (c-Fos) did not change the observed binding pattern. The anti-p50 antiserum diminished the intensity of the lower specific DNA/protein complex only, whereas the p65/RelA

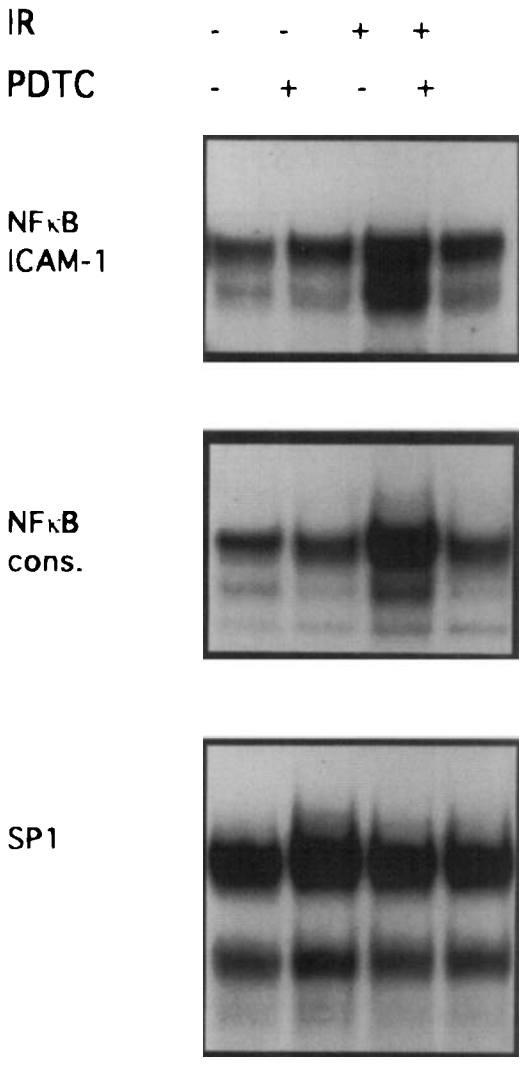

FIGURE 5 **IR induces binding activity at an NFκB-like site in the 5′ region of the human ICAM-1 gene, and this induction is inhibited by PDTC.** Nuclear extracts were prepared from HaCaT cells that were either not irradiated or irradiated with 20 Gy of IR 1 h prior to nuclear extract preparation. Some cells were also incubated with 100 μM PDTC 30 min prior to the time point of irradiation. Extracts were incubated with a ^{32}P-labeled double stranded oligonucleotide representing genomic ICAM-1 sequences (positions –199/–170 relative to the transcription start site including the NFκB-like binding motif 5′ CGGAATTTCC 3′; Table I); a ^{32}P-labeled double stranded oligonucleotide containing an NFκB consensus motif; or a ^{32}P-labeled double stranded oligonucleotide containing an SP1 consensus motif (Table I). Incubation was followed by electrophoresis on a non-denaturing polyacrylamide gel.

antiserum disrupted both complexes and also produced a supershifted complex. Preincubation with antisera specific for other members of the NFκB-family including c-Rel, RelB (Fig. 7), and p52/NFκB-2 (not shown) did not affect the DNA/protein complexes. This constellation is consistent with a combination of a p50-p65/RelA heterodimer (lower retarded band) and an p65/p65 homodimer (upper retarded band).

Upstream NFκB-like Sequences Show Complex Formation, But are Not Induced in Response to IR

In further electrophoretic mobility shift assays, a putative upstream NFκB-like binding site (5′ GGGAGGATTCC 3′) at positions –528/–519[14] was analyzed with respect to IR-inducibility. Using a double-stranded oligonucleotide fully containing this NFκB-binding site motif (Table I) resulted in the retardation of protein/DNA complexes, but this binding activity was not different in extracts from either untreated or IR-stimulated cells (data not shown). Moreover, the binding complexes did not appear to be sequence specific for NFκB, because they could not be competed away by an excess of an unlabeled double-stranded oligonucleotide containing an NFκB consensus binding motif. Additionally, PDTC did not inhibit binding activity in extracts from both irradiated and unirradiated cells. Therefore, by both deletional transcription analysis and by electrophoretic mobility shift assay, the NFκB binding motif at positions –528/–519 is not likely to be involved in ICAM-1 gene induction by IR in the investigated epithelial cell lines.

DISCUSSION

In this study we have elucidated molecular mechanisms that lead to the induction of ICAM-1 expression by IR. We have also shown that IR-induced ICAM-1 transcription and surface protein expression can be inhibited by antioxidants.

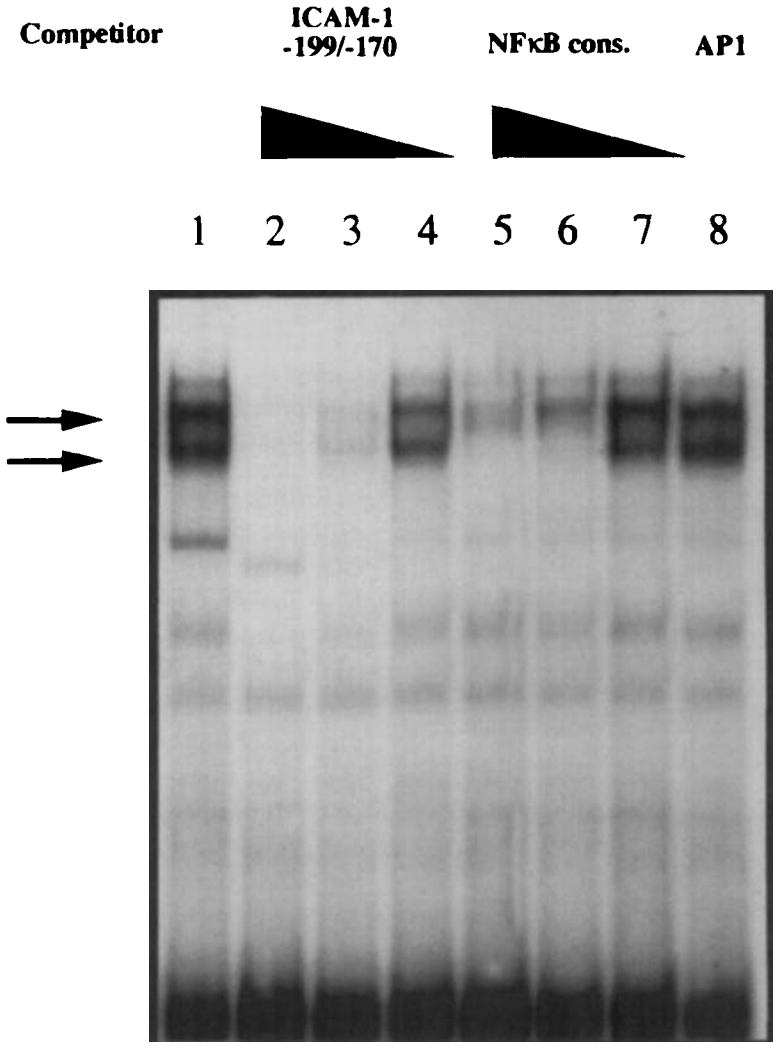

FIGURE 6 **The IR-induced binding activity is NFκB-specific**. Nuclear extracts were prepared from HaCaT cells treated with 20 Gy of IR 1 h prior to extract preparation. Extracts were incubated with a ^{32}P-labeled double stranded oligonucleotide representing genomic ICAM-1 sequences (positions –199/–170 including the NFκB-like binding motif 5′ CGGAATTTCC 3′; Table I). Incubation was followed by electrophoresis on a non-denaturing polyacrylamide gel. Specific binding complexes are marked by arrows. Unlabeled identical oligonucleotide was added in varying amounts (lane 2: 50 ng, lane 3: 5 ng, lane 4: 0.5 ng). In lane 6–8, an unlabeled double stranded oligonucleotide encompassing an NFκB consensus motif (Table I) was added (lane 5: 50 ng, lane 6: 5 ng, lane 7: 0.5ng). In lane 8, an unlabeled double stranded oligonucleotide containing an AP1 binding motif (irrelevant DNA-sequence, 50 ng) was added.

The analysis of 5′ deleted ICAM-1-based CAT constructs suggests that a DNA segment between positions –277 and –182 is involved in IR-induction of ICAM-1. Consistent with this finding, an IR-inducible, PDTC-inhibitable NFκB-like binding motif (at –186/–177) was demonstrated in electrophoretic mobility shift assays.

The transcription factor NFκB is composed of a heterodimer of structurally related DNA-binding protein subunits.[30] In its inactive state, NFκB is located in the cytoplasm associated with inhibitory proteins of the IκB-protein family. The preformed heterodimers are released from IκB by a large number of stimuli including PMA, TNF-α,

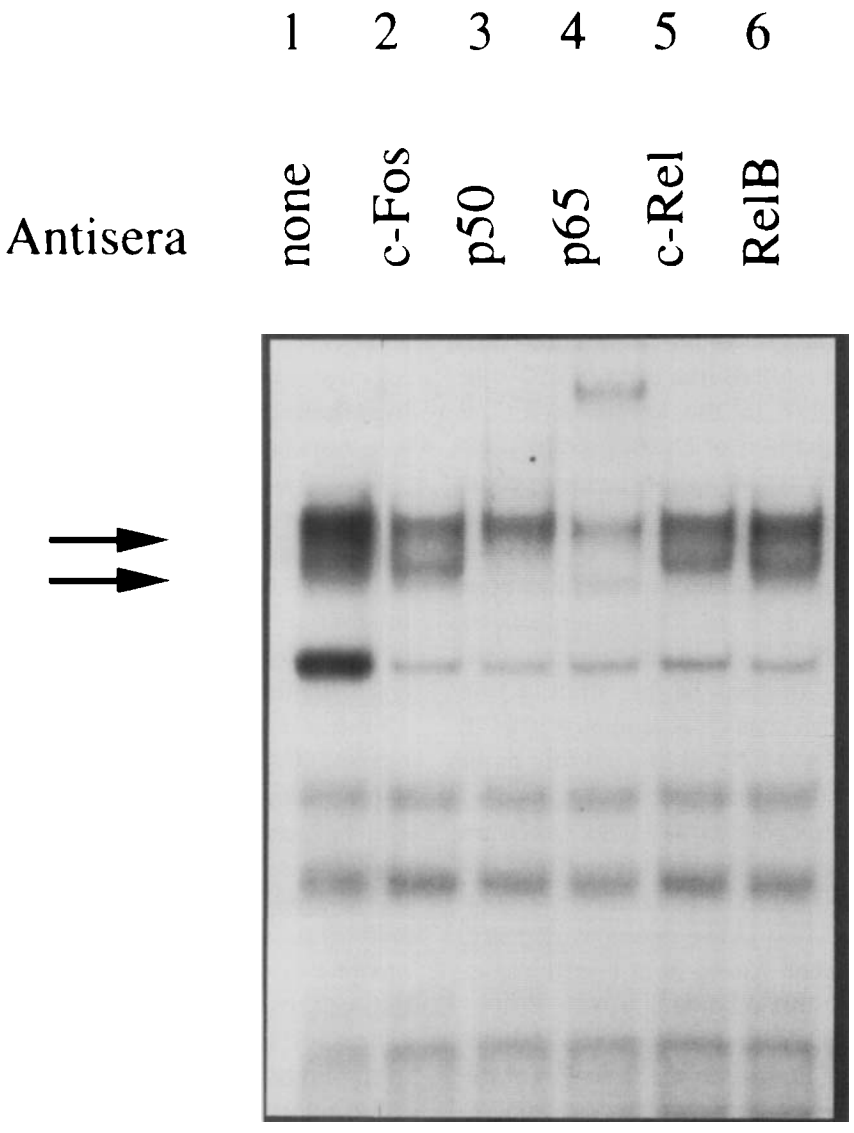

FIGURE 7 **Antisera against p50 or p65/RelA interfere with IR-inducible complex formation at the NFκB-like binding site.** Nuclear extracts prepared from HaCaT cells treated with 20 Gy of IR 1 h prior to extract preparation were incubated with a ^{32}P-labeled double stranded oligonucleotide representing genomic ICAM-1 sequences (positions –199/–170 including the NFκB-like binding motif 5′ CGGAATTTCC 3′; Table I). Incubation was followed by electrophoresis on a non-denaturing polyacrylamide gel. Specific binding complexes are marked by arrows. Prior to addition of the double stranded oligonucleotide, extracts were incubated with polyclonal antisera specific for various members of the NF'B-family (p50, lane 3; p65 /RelA, lane 4; c-Rel, lane 5; RelB, lane 6). As controls lane 1 is a reaction without addition of antiserum and lane 2 represents an extract incubated with an antiserum against c-Fos, a transcription factor that does not bind to sequences present in the used double stranded oligonucleotide probe.

and IL-1, all of which probably induce proteolytic degradation of IκB. Released NFκB is translocated to the nucleus, where it binds to specific DNA-motifs and interacts with the transcription complex. Besides the initially described subunits p50 and p65/RelA, other structurally related proteins have been identified as possible constituents of the dimeric transcription complex, including p52/NFκB2, c-Rel, and RelB.[31]

In supershifts we have identified p50 and p65/RelA as constituents of the IR-induced binding complex at the NFκB-like binding motif at positions −186/−177 in the keratinocyte cell line HaCaT. The pattern of DNA/protein complex disruption by transcription factor specific antisera was consistent with the combination of a p50-p65/RelA heterodimer and a p65/p65 homodimer. A similar binding pattern has been observed at this motif in TNF-α stimulated extracts of HUVEC.[17] These findings are also supported by an analysis of the binding fine specificities of NFκB sites.[32] A sequence 10 of 10 identical to the ICAM-1 NFκB-like binding motif at positions −186/−177 has been shown to bind p65- or c-Rel, but not p50 homodimers, which are usually found at the classical NFκB motif.[30] The binding pattern at the NFκB-like motif at positions −186/−177 may show tissue variability, since c-Rel has been found as a binding constituent in a melanoma cell line.[33]

We describe for the first time that IR induces NFκB like binding activity in the 5′ region of the human ICAM-1 gene in extracts of the keratinocyte derived HaCaT cell line. The IR-inducible NFκB-like binding motif (at −186/−177) has previously been implicated in ICAM-1 transcriptional induction by phorbol esters and TNF-α.[17,19–21] We also demonstrate the activation of NFκB by IR at the classical NFκB motif derived from HIV-1 long terminal repeats, which confirms previous reports in the myeloid leukemia cell line KG-1[29] and the lymphoblastoid cell line 244B.[34] IR produces reactive oxygen intermediates,[25] and reactive oxygen intermediates activate NFκB.[35] The inhibition of IR-induced NFκB binding activity by antioxidants suggests that ICAM-1 induction by IR is mediated by the production of reactive oxygen intermediates and subsequent activation of NFκB in a keratinocyte environment. The prevention of NFκB activation by antioxidants has previously been reported.[36,37] While the molecular mechanisms are not totally clear, it is currently thought that antioxidants inhibit the phosphorylation and subsequent degradation of IκB by inhibiting one or several IκB kinases.[31] Our findings with a strong pro-oxidant stimulus (IR) and the neutralization of its effects by antioxidants further support the hypothesis that NFκB is an oxidative stress responsive transcription factor.[38]

Several reports have previously indicated that ICAM-1 expression may be influenced by the redox state of the cell. In cultured human keratinocytes ICAM-1 expression was induced by incubation with H_2O_2, and this induction was inhibited by antioxidants.[39] Additionally, some antioxidants also blocked ICAM-1 induction by TNF-α and IFN-γ.[39] In human fibroblasts, PDTC blocked ICAM-1 induction by IL-1 and IFN-γ.[40] Our finding that IR-induced ICAM-1 mRNA- and cell surface expression is inhibited by antioxidants supports the view that IR induces ICAM-1 mRNA- and cell surface expression by an increase in intracellular reactive oxygen intermediates. Antioxidants antagonized not only IR-induced, but also constitutive ICAM-1 surface expression in HeLa cells (Fig. 3) suggesting that in certain tissues ICAM-1 expression mechanistically reflects the redox state of the cell. In endothelial cells (HUVEC), however, PDTC did not inhibit TNF-α, IL-1β, or LPS induced ICAM-1 mRNA or cell surface expression, and it was concluded that in this cell type ICAM-1 was not influenced by the redox state of the cell.[41] In another study of HUVEC cells, coadministered PDTC prolonged, but did eventually not prevent TNF-α mediated ICAM-1 induction.[42] The molecular basis for this tissue-related difference in sensitivity of ICAM-1 expression to reactive oxygen intermediates is yet to be clarified, but might reflect tissue specific differences in ICAM-1 tran-

scriptional control. Tissue-specific differences in constitutive and induced ICAM-1 transcription have previously been observed,[43,44] and tissues displaying PDTC-inhibitable and PDTC non-inhibitable ICAM-1 expression can be separated in as much as the former (keratinocytes and fibroblasts) have a low or absent constitutive ICAM-1 expression and the latter (endothelial cells) constitutively express significant amounts of ICAM-1.

A putative upstream NFκB-like binding site at positions –528/–519 relative to the transcription start site[14] displayed DNA/protein complex formation which, however, appeared to be neither NFκB-specific nor IR-inducible in HaCaT nuclear extracts. Similarly, we have previously observed constitutive binding, but no induction of binding by the phorbol ester PMA, at this motif in the squamous carcinoma cell-derived cell line A431.[21] Others have observed inducible NFκB-like binding[40] at a neighboring motif at positions –540/–531[15] in response to IL-1α in human fibroblasts. In the same study, mutation or 5′ elimination of the NFκB motif abrogated PDTC-mediated inhibition of IL-1α induction of ICAM-1 promoter-CAT constructs, and it was suggested that this site may be critical for PDTC inhibition of ICAM-1 induction.[40] However, the functional significance of this observation for ICAM-1 transcription is unclear, because in promoter studies the gene segment around the –540/–531 NFκB binding site was not required for induction of transcription by the pro-oxidant stimulus IL-1α.[40] Using IR as a different pro-oxidant stimulus, we have also found no evidence for a prominent functional role for the gene segment around the upstream NFκB binding site, nor have several previous studies employing various investigative stimuli including cytokines (IL-1α, TNF-α, IFN-γ)[17,20,22,23,33] and phorbol esters.[19,21]

The observed IR-mediated induction of promoter activity in the –277/+1-ICAM-1-CAT construct is quantitatively comparable to the transcriptional induction observed in a different CAT-linked promoter (c-Jun promoter) after exposure to the same IR-dose (20 Gy).[45] The IR-mediated induction of ICAM-1 cell surface expression in HeLa cells was statistically significant and comparable to the IR-mediated twofold induction of ICAM-1 cell surface expression observed in the keratinocyte cell line HaCaT.[11] On the other hand, a stronger (up to tenfold) induction of ICAM-1 cell surface expression was observed in HL60 cells under otherwise identical experimental conditions.[11] Differences between cell lines with regard to the extent of IR-inducibility have previously been observed with several other genes.[46] These differences may be due to different cellular reductive capacities which may reflect a different cellular glutathione content. In support of this view, using a different pro-oxidant stimulus (UVA1 radiation), ICAM-1 cell surface expression could be induced in cultured keratinocytes, but not in a transformed keratinocyte cell line with a threefold higher content of glutathione. However, when the glutathione level of the transformed cell line was lowered, it became susceptible to UVA1-mediated ICAM-1 induction.[47]

Comparatively high single doses were applied to cells in order to demonstrate the effects of IR on ICAM-1 expression unambiguously. Similar doses have been utilized to investigate the influence of IR on the expression of other molecules.[29,48] However, in a skin organ culture model[11] we have also observed ICAM-1 induction with lower dose (6 Gy) that is within the range applied as single dose in dermatological radiotherapy (2–8 Gy).[49] This suggests that cutaneous ICAM-1 induction may play a role in the development of acute radiodermatitis, routinely observed in dermatological radiotherapy. ICAM-1 might also be upregulated by ionizing total body irradiation prior to bone marrow transplantation. In such a circumstance, ICAM-1 induction may enhance adherence and subsequent allogeneic activation of donor T-cells in the host. Thus, the beneficial effect of anti-ICAM-1 MoAb during the course of semiallogeneic bone marrow transplantation in mice[50]

may in part be due to a reduced preactivation of the host by conditioning total body irradiation.

In summary, our data suggest that ICAM-1 induction by IR is mediated by the production of reactive oxygen intermediates. The IR induced, PDTC-inhibitable binding activity at the ICAM-1 NFκB binding site is consistent with the view that NFκB is involved in the IR induction of ICAM-1.

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