Insulin-like Growth Factor-I (IGF-I) Receptor Activation Rescues UV-damaged Cells through a p38 Signaling Pathway

POTENTIAL ROLE OF THE IGF-I RECEPTOR IN DNA REPAIR*

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The activated insulin-like growth factor-I receptor (IGF-IR) is implicated in mitogenesis, transformation, and anti-apoptosis. To investigate the role of the IGF-IR in protection from UV-mimetic-induced DNA damage, 4-nitroquinoline N-oxide (4-NQO) was used. In this study we show that the activation of the IGF-IR is capable of rescuing NWTb3 cells overexpressing normal IGF-IRs from 4-NQO-induced DNA damage as demonstrated by cellular proliferation assays. This action was specific for the IGF-IR since cells expressing dominant negative IGF-IRs were not rescued from 4-NQO UV-mimetic treatment. DNA damage induced by 4-NQO in NWTb3 cells was significantly decreased after IGF-IR activation as measured by comet assay. IGF-I was also able to overcome the cell cycle arrest, observed after 4-NQO treatment, thereby enhancing the ability of NWTb3 cells to enter S phase. Interestingly, the p38 mitogen-activated protein kinase pathway was shown to represent the main signaling pathway involved in the IGF-IR-mediated rescued of UV-like damaged cells. The ability of the IGF-IR to induce DNA repair was also demonstrated by infecting NWTb3 cells with UV-irradiated adenovirus. Activation of the IGF-IR resulted in enhanced β-galactosidase reporter gene activity demonstrating repair of the damaged DNA. This study indicates a direct role of the IGF system in the rescue of damaged cells via DNA repair.

The insulin-like growth factors (IGF-I, IGF-II) belong to a pleiotropic family of soluble peptide factors that circulate bound to one of six IGF-binding proteins. The IGFs, their receptors, and IGF-binding proteins constitute a family of cellular modulators that play essential roles in the regulation of growth and development. The actions of IGFs are believed to result primarily from the activation of the IGF-I receptor (IGF-IR) (1). The IGF-IR resembles the insulin receptor in structural as well as functional aspects (2). This heterotetrameric transmembrane glycoprotein consists of two α- and two β-subunits. The tyrosine kinase catalytic site and the ATP-binding site are located in the cytoplasmic portion of the β-subunit. The β-subunit of the IGF-IR has intrinsic tyrosine kinase activity that is stimulated when IGF-I binds to the α-subunit. The receptor tyrosine kinase phosphorylates intracellular substrates such as the insulin receptor substrates IRS-1, IRS-2, and Shc. Two different major pathways then emerge; one pathway involves ERK1/2 through Ras/Raf/MEK and the other pathway proceeds through PI3K (see for review Ref. 1). Thus, the IGF-IR activated by its ligands plays an important role in the growth of cells inducing mitogenesis, transformation, and protection of cells from a variety of apoptotic injuries (3). The IGF-IR was also shown to be involved in mitogenesis through the IRS molecules (4), and IGF-I can also protect fibroblasts from apoptosis induced by UV-B light through the activation of the phosphatidylinositol 3′-kinase (PI3K)/Akt pathway (5). Moreover, the MAPK pathway may play a role in cell survival independent of PI3K/Akt as was demonstrated in fibroblasts overexpressing the IGF-IR (6, 7). IGF-I can also stimulate the proliferation of hematopoietic cells in combination with other hematopoietic growth factors (8), and IGF-I is known to support growth and to prevent apoptosis in neuronal cells (9, 10). The viability of most cells is dependent on the presence of growth factors; recently, IGF-IR-mediated neuroprotection was shown to be mediated via induction of NF-κB that is dependent upon activation of the PI3K pathway (11). Keratinocytes are more susceptible to UVB-induced apoptosis if the growth medium is depleted of exogenous growth factors; the activation of the IGF-IR was shown to promote the survival of human keratinocytes following UVB irradiation (12).

To investigate further the mechanisms involved in IGF-IR activation on rescuing cells against UV-like DNA damage, different cell lines were damaged with 4-NQO, an UV-like mimetic agent. We used NWTb3 cells, a NIH-3T3-derived cell line overexpressing the normal human IGF-IR (13). To compare cell rescue of NWTb3 cells, parental NIH-3T3 and NIH-3T3-derived cell lines expressing dominant negative IGF-IRs (NKA8 and NKR1) were used (14). We investigated the effect of IGF-IR stimulation on the cellular proliferation of cells after DNA damage, and we determined which signaling pathways are involved in this cell rescue. With the use of an UV-damaged adenoviral vector, we also show the direct effect of IGF-IR activation on DNA repair.
EXPERIMENTAL PROCEDURES

Materials—4-Nitroquinoline 1-oxide (4-NQO) and P38 kinase-specific inhibitor LY 294002 were purchased from Sigma, and recombinant human IGF-I (rhIGF-I) was a gift of Genentech (San Francisco, CA). Both MEK1/2-specific inhibitor U 0126 and p38 MAP kinase-specific inhibitor SB 202190 were from Calbiochem. The stock solutions of pharmacological inhibitors such as LY 294002 and U 0126, and SB 202190 were in MeSO at a concentration of 1000-fold so that when they were added to the culture medium, the concentration of MeSO was below 0.1%.

The antibodies used for Western blotting were purchased from New England Biolabs Inc. (Beverly, MA); they include phospho-Akt (Ser-473), phospho-MAPK (ERK1/2) (Thr-202/Tyr-204) and phospho-p38 MAPK (Thr-180/Tyr-182) polyclonal antibodies as well as the Akt, MAPK, and p38 protein-specific antibodies and the anti-rabbit IgG secondary antibody (horseradish peroxidase-conjugated). Cellular culture media and reagents were purchased from Life Technologies Inc. The p38 dominant negative construct (pCMV5-p38AGF) was kindly provided by Dr. R. J. Davis (Howard Hughes Medical Institutes, Worcester, MA); the “G” motif of the p38 MAPK (phosphorylation site) was replaced by AGF, thereby preventing p38 MAPK phosphorylation. AdCA35Stc in replication-deficient recombinant type 5 adenovirus expressing the lacZ gene under the control of the mouse cytomegalovirus (MCMV) immediate early promoter (15) was kindly provided by Dr. F. Graham from the McMaster University, Hamilton, Ontario, Canada.

Cell Culture—The parental mouse embryonic fibroblast NIH-3T3 cell line expresses 105 receptors/cell (13). The NWTb3 cell line established by Dr. F. Graham expresses the human IGF-I receptor of ~4 x 105 receptors/cell (13). The NKA8 and NKR1 cell lines, where the Lys-1003 residue at the ATP-biding site was substituted by Ala (NKA8 mutant) or Arg (NKR1 mutant), respectively (14), express the dominant negative human IGF-IR (~3–7 x 105 receptors/cell). The parental NIH-3T3 cell line was routinely cultured in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% heat-inactivated fetal bovine serum (FBS), 100 units/ml penicillin, 100 μg/ml streptomycin, and 300 μg/ml g-t-glutamyl-tarcidin in a humidified atmosphere of 95% air and 5% CO2 (37 °C). NWTb3, NKA8, and NKR1 cells were cultured in the same medium with the addition of 0.5 g/liter G418 (Geneticin).

Immunoblotting—NWTb3 cells were cultured in 100-mm dishes (1 x 106 cells/dish) in the medium described under the cell culture method. The day after seeding, cells were starved overnight in FBS-free medium. After incubation (1 h at 37 °C) with inhibitors (appropriate dilutions) and stimulation with 50 nM IGF-I (10 min at 37 °C), the cells were washed with ice-cold PBS, and total cell lysates were prepared on ice with lysis buffer (20 mM Tris, pH 7.5, 100 mM NaCl, 2.5 mM EDTA, 1% Triton X-100, 0.5% deoxycholate, 0.1% SDS, 5% glycerol, 10 mM NaF, 0.3 mM NaMoO4, 1 mM NaNO3, and 0.5 mM 4-(2-aminoethyl)-benzenesulfonyl fluoride hydrochloride). The cell lysates were centrifuged for 30 min at 15,000 g, the supernatant was collected, and the total protein concentration was measured using the BCA protein assay kit (Pierce). Phosphorylated Akt, ERK1/2, and p38 as well as Akt, ERK1/2, and p38 protein were detected in 15 μg of protein cell lysate by Western blotting using antibodies against phospho-Akt, phospho-ERK1/2, and phospho-p38 as well as against total Akt, ERK1/2, and p38 protein, respectively. All antibodies used were diluted 1:1000 in Tris-buffered saline/Tween 0.1% plus 5% bovine serum albumin.

4-NQO Treatment—The UV-mimetic drug 4-NQO was dissolved in absolute ethanol at a concentration of 2 mM, whereupon a 100 μM stock solution was prepared by diluting the concentrated solution in serum-free medium and stored at −20 °C for use throughout the study. All 4-NQO treatments were performed with a final concentration of 2.5 μM 4-NQO in serum-free medium for 30 min at 37 °C in a CO2 incubator. The following 4-NQO treatment, cultures were rinsed with pre-warmed (37 °C) 5% FBS/DMEM and then incubated in the same medium containing different compounds (IGF-I and specific pathway inhibitors) for various times as indicated in the cell proliferation method.

Cell Proliferation—The cell proliferation assay was performed using the CyQUANT Cell Proliferation kit (Molecular Probes, Eugene, OR) and the TACS7000 BioAssay reader (PerkinElmer Life Sciences). The NWTb3 overexpressing IGF-IR and the other cell lines were cultured in 75-cm2 flasks. When flasks were 90% confluent, cells were counted using a hemocytometer. Cells were seeded on 96-well Costar Cluster plates (Corning Glass) in 200 μl of DMEM with 10% FBS. For the standard curve, the cell number at base line ranged from 1,000 to 40,000 per well. After 15 h of incubation, the medium was changed to 1% FBS/DMEM for an overnight starvation. 2.5 μM 4-NQO was added to the medium during 30 min at 37 °C, and cells were then washed and cultured in 5% FBS/DMEM with or without 50 nM IGF-I, in the absence or in the presence of different inhibitors (see figure for the concentrations used). Cells were then incubated for 5 days. The same experiment was performed on NWTb3 cells transiently transfected with a dominant negative p38 construct (pCMV5-p38AGF). The transfection was performed the day after seeding the cells, and cell proliferation assay was performed as described, with 4-NQO and IGF-I treatments. At the indicated times, the medium from plates was aspirated, and the plate was stored at −70 °C until all plates had been processed. The cells were then lysed using the lysis buffer from the CyQUANT kit. The fluorescence intensity of the DNA binding dye was measured using an HTS 7000 microplate reader with excitation at 485 nm and emission at 535 nm.

Transient Transfection of p38 Dominant Negative Construct (pCMV5-p38AGF)—NWTb3 cells were seeded the day before the transfection. Cells were then transiently transfected using the FuGEN6 transfection reagent (Roche Molecular Biochemicals) according to the manufacturer’s instructions with 3.6 μg of a p38 dominant negative construct (16) or vector alone (p38 dn +) or vector alone (−). In order to determine the transfection efficiency under the conditions used in this experiment, 3.6 μg of DNA from pSw-β-galactosidase control vector (Promega, Madison, WI) was used to transiently transfect NWTb3 cells. 24 h after transfection, in situ β-galactosidase staining for enzyme expression was performed, and transfected cells (blue staining) were counted as the total cell number. To check p38 phosphorylation in NWTb3 cells transfected with the p38 dn construct and compare with cells transfected with vector alone, cells were starved overnight 24 h after transfection and stimulated with 50 nM IGF-1 (10 min at 37 °C). Protein extracts were prepared as described previously, and Western blot analyses were performed with anti-phospho-p38 antibody to detect p38 phosphorylation induced by IGF-I stimulation as well as with a p38 antibody to detect both endogenous and transfected p38 proteins (transfection efficiency).

Comet Assay—A single cell gel electrophoresis assay (17) was performed using the Trevigen Comet assay kit (Trevigen, Gaithersburg, MD) according to the manufacturer’s conditions. This comet assay provides an effective method for evaluating DNA damage into cells. The principle of this assay is based upon the ability of denatured and cleaved DNA fragments to migrate out of the cell under the influence of an electric potential, whereas undamaged and supercoiled DNA stays in the cell when a current is applied.

NWTb3 cells (190,000 cells/well) were plated in 6-well plates in 10% FBS/DMEM. After seeding (15 h), the medium was replaced by 1% FBS/DMEM for overnight starvation. Cells were treated or not with 2.5 μM 4-NQO during 30 min at 37 °C. Cells were then treated with or without 50 nM IGF-1 in DMEM, 5% FBS for the indicated times. Untreated cells (negative controls) and cells treated with 100 μM H2O2 (positive controls) were included in the assay. At every 24 h time point, cells were scraped and counted. After washing once with ice-cold PBS, a dilution of 1 x 105 cell/ml was used to perform the assay. Cells were mixed with molten agarose (ratio 1:10), 38 μl were pipetted on glass slides. Slides were immersed in lysis solution and incubated on ice for 30 min, followed by an immersion in an alkaline buffer for 1 h at room temperature in the dark. Slides were rinsed in TBE 1X before electrophoresis (1 V/cm for 10 min). Samples were stained with the supplied SYBR Green dye, and slides were viewed using an inverted fluorescence microscope equipped with a FITC filter (AXIOVERT 1005, Zeiss). The presence of a tail, so called “comet,” reflects DNA damage (single- and double-strand breaks, alkali-labile sites). For each condition, “comet cells” were counted in a total of 200 cells.

Flow Cytometry—Every 48 h following treatment with 2.5 μM 4-NQO (in control experiments 4-NQO was omitted) and incubation with or without 50 nM IGF-I in the presence of 5% FBS/DMEM, cells were collected and washed twice in PBS. Cell pellets were then resuspended in 100 μl of PBS, fixed in 1 ml of 70% ethanol, 30% saline buffer, and stored at −20 °C until analysis. Cells were washed twice with PBS followed by an incubation for 40 min in 0.5 ml of PBS containing 0.1% Triton and 50 μg of RNase at room temperature. 20 μg of propidium iodide (Sigma) was added, and the suspension was incubated in the incubator at room temperature for 15 min. DNA content staining was finally determined by flow cytometry. All analyses were carried out on a FACSCalibur using CellQuest Software (Becton Dickinson, Mountain View, CA).

Adenovirus Amplification, Purification, and Titration—The recombinant adenovirus AdCA55La2 used in this study was constructed as described previously (15). In this vector, the Escherichia coli lacZ reporter gene is under the control of a 1.4-kilobase pair MCMV immediate
early promoter. The virus was propagated in human 293 cells grown in modified Eagle’s medium enriched with l-glutamine (2 mM), penicillin (100 units/ml), streptomycin (0.1 mg/ml), and 10% heat-inactivated FBS. AdCA35lacZ was amplified and purified as described previously (18). Briefly, purified adenovirus was obtained from a crude viral lysate by purification on a CaCl2 gradient resulting in a titer of 4 × 108 plaque-forming units (pfu/ml). 100-μl aliquots of the adenovirus were frozen at −70 °C and kept until use.

UV Irradiation of the Virus—UV irradiation of the virus has been described previously (19). Briefly, 40 pfu/cell of viral suspension, diluted in 1% FBS/DMEM, was irradiated in a 35-mm dish on ice with continuous stirring using an EL UV lamp (model UVLS-28, 254 nm) and a UVX radiometer (both from UVP, Inc., Upland, CA) at an incident fluence rate of 2 wats/m2. For the host cell reactivation curve (HCR), the UV dose rates used were 0, 100, 200, 300, 500, 800, and 1000 J/m2. Aliquots of 200 μl were removed for each exposure to the virus and diluted appropriately with 1% FBS/DMEM.

IGF-I Treatment of the UV-irradiated Reporter Gene—NWTb3 cells were seeded in 6-well plates at a density of 190,000 cells/well. 20 h after seeding, the medium was aspirated, and cells were infected with either UV-irradiated or non-irradiated AdCA35lacZ in a volume of 300 μl at a multiplicity of infection (m.o.i.) of 40 pfu/cell. Following viral adsorption for 90 min at 37 °C, the cells were washed twice with medium and re-fed with 2 ml of warmed 1% FBS medium. For the IGF-I experiments on the damaged 90-min infection, the cells with the UV-irradiated virus was followed by washing the cells to remove any trace of virus, and cells were then re-fed with 1% FBS/DMEM with or without 50 nm IGF-I. Infected cells were harvested 48 h after infection, and cells were resuspended in 400 μl of reporter lysis buffer (Promega, Madison, WI). Transgene activity was measured using the β-galactosidase assay kit (Promega). Results were normalized, and β-galactosidase activity of the cells infected with non-irradiated virus (1/m2) was set at a level of 1. Similar values were obtained when cell number was used for normalization.

RESULTS

NWTb3 Rescue Induced by IGF-IR Activation after 4-NQO Treatment—Given the importance of IGF-I and IGF-IR pathway in cell proliferation (4, 20), we examined the effect of IGF-IR activation on the cellular proliferation of mouse embryonic fibroblasts upon an UV-mimetic treatment using 4-NQO. Cell proliferation assays were performed on NWTb3 cells over-expressing normal human IGF-IR (13). NWTb3 cells were treated with 4-NQO for 30 min at a concentration of 2.5 μM that was determined to induce cell growth arrest. 4-NQO was removed after the treatment, and the cells were washed twice and then stimulated with IGF-I at a physiological concentration (50 nm). As expected, IGF-I induced the stimulation of NWTb3 cell proliferation with an increasing cell number that reached a plateau after 4 and 5 days when cells became confluent (Fig. 1A). Moreover after 4-NQO treatment, IGF-IR activation resulted in an enhanced cellular proliferation of NWTb3, i.e. IGF-I was able to rescue the cells, whereas no growth was observed in the absence of IGF-I. This effect of IGF-I could be detected as early as 3 days after the 4-NQO treatment and was significant at 4 and 5 days (p < 0.001). Not only was IGF-I capable of maintaining the survival of these cells, but in addition an obvious enhancement of the cellular proliferation could be observed 4 and 5 days after 4-NQO treatment. The same experiment was performed with NIH-3T3 parental cells (Fig. 1B) and two cell lines expressing the dominant negative human IGF-IR, NKA8 and NKR1 cells ADDIN ENR(m) (14) as shown in Fig. 1, C and D, respectively. It has been shown that NIH-3T3 cells express ~16 000 IGF-IR/cell as compared with the NWTb3 cells (~4 × 106 IGF-IR/cell). The IGF-I rescue was observed only in NWTb3 cells over-expressing normal IGF-IRs as compared with parental NIH-3T3s, NKA8, and NKR1 cell lines. Thus, these data demonstrate that IGF-IR activation can overcome the inhibition of the cellular proliferation induced by 4-NQO, an UV-mimetic drug.

4-NQO Cell Cycle Arrest Is Overcome by IGF-I—In order to determine the role of 4-NQO in inhibiting cellular proliferation and the effect of IGF-I in overcoming this inhibition, we carried out fluorescence-activated cell sorter analysis. We examined the DNA content of cells treated (or not) with 2.5 μM 4-NQO and stimulated (or not) immediately after the 4-NQO with 50 nm IGF-I. 4-NQO induced cell cycle arrest of NWTb3 cells in G0–G1 since the percentage of cells in S phase was low and most of the cells were present in G0–G1 (Table I). No sub-2N (apoptotic) cells were detected in the presence of 4-NQO. IGF-I treatment was capable of rescuing the cells from growth arrest induced by 4-NQO as shown by an increase in S phase from 9 to 15% at day 2 and from 6 to 23% at day 4 after 4-NQO treatment (Table I). As a control experiment, DNA content analysis was performed on NWTb3 cells not treated with 4-NQO 2 and 4 days after the addition, or not, of IGF-I. As shown in the Table I (see numbers in parentheses), IGF-I was not able to increase significantly the number of cells in S phase (10–12% on day 4 after IGF-I treatment). These findings support the results obtained from the cellular proliferation assay of NWTb3 cells in the presence of 4-NQO and IGF-I.

IGF-I Decreases DNA Damage following 4-NQO Treatment—NWTb3 cells were exposed to the DNA-damaging agent 4-NQO, with the aim of determining whether DNA damage occurring upon 4-NQO treatment could be restored by IGF-IR activation. Cell DNA damage was detected using a “comet assay” (also called single cell gel electrophoresis). To perform such an assay, NWTb3 cells were treated with 2.5 μM 4-NQO as described under “Experimental Procedures” and incubated with medium, with or without 50 nm of IGF-I, up to 5 days after the 4-NQO treatment. The cells exhibiting DNA damage, named comet cells were counted randomly for a total of 200 cells. As shown in Fig. 2, about 35% of the cells were shown to be damaged (comet cells) when the experiment was performed 4 days after the 4-NQO treatment, and 40% of the cells were comet cells 5 days after the same treatment. At each time point, IGF-I was able to decrease the percentage of comet cells from 35 (day 4) or 40% (day 5) to 5%. As shown in the figure, the level of DNA-damaged cells following the addition of IGF-I reaches the level of undamaged cells (negative control). Thus, it appears that these results correlate with those obtained with the cell proliferation assay; IGF-I is involved in rescuing NWTb3 cells from DNA damage, leading to normal cellular proliferation.

p38 MAP Kinase Is Involved in IGF-I-Induced Cell Proliferation following DNA Damage—In order to study the signaling pathways involved in the effect of IGF-I to rescue NWTb3 cells damaged following 4-NQO treatment, PI3K-, ERK1/2-, and p38-specific inhibitors were used. NWTb3 cells were treated with 2.5 μM 4-NQO and incubated during the indicated periods in the absence or in the presence of 50 μM IGF-I to induce rescue. Fig. 3 indicates that in the presence of the PI3K pathway inhibitor LY 294002 (3A) or in the presence of the ERK1/2 inhibitor U 0126 (3B), no effect of these inhibitors was observed on the IGF-I rescue. In order to ensure that the inhibitors were active on protein phosphorylation induced by IGF-I stimulation, NWTb3 cells were treated for 1 h with inhibitors prior to the IGF-I stimulation (10 min). Western blotting analysis (Fig. 4) revealed the dose-dependent inhibition of LY 294002 (PI3K pathway), U 0126 (ERK1/2 pathway), and SB 202190 (p38 pathway), respectively, on the IGF-I-induced Akt, ERK1/2, and p38 phosphorylation. As shown in Fig. 4A, 10 μM LY 294002 was able to inhibit Akt phosphorylation induced by IGF-I stimulation and in the same manner; 2 μM U 0126 and 50 μM SB 202190 inhibited ERK1/2 (Fig. 4B) and p38 (Fig. 4C) phosphorylation, respectively. Western blot analyses, performed to detect all forms of Akt, ERK1/2, and p38 kinases, revealed that protein levels were similar among the samples.
Since inhibition of IGF-I-induced rescue of cells could not be detected with the use of PI3K and ERK1/2 pathway inhibitors, we employed a specific p38 MAP kinase inhibitor SB 202190, which fully inhibits p38 kinase activity without affecting JNK or ERK activities (21). Most interesting, the addition of SB 202190 significantly inhibited the IGF-I rescue of NWTb3 cell proliferation (Fig. 3C). Four to 5 days after the 4-NQO treatment, IGF-I induced cellular proliferation to a level of 161 ± 6 and 166 ± 4%, respectively, above cells not receiving IGF-I indicating rescue from DNA damage. In the presence of SB 202190 cell numbers were respectively reduced to 118 ± 4 and 122 ± 3% above untreated cells. When inhibitors of the different signaling pathways were combined, there was total abrogation of the IGF-1 rescue. Thus the rescue of NWTb3 cells induced by IGF-1R activation seems to be mediated primarily through the p38 MAPK pathway.

In order to confirm this result, NWTb3 cells were transiently transfected with a dominant negative p38 construct (p38 dn). Upon IGF-I stimulation, there was a 60% reduction in p38 phosphorylation after transfection of the dominant negative construct (data not shown). Cell proliferation assays were performed on NWTb3 transfected with p38 dominant negative (+) or empty vector (−) as a control (Fig. 5). After induction of 4-NQO-DNA damage and upon IGF-I stimulation, transfected cells with empty vector could be rescued from DNA damage and started to grow upon IGF-I treatment after 4 and 5 days. However, when NWTb3 cells were transfected with a dominant negative p38 MAPK, the IGF-I rescue was markedly reduced. As observed in the Fig. 5, the inhibition of the IGF-1 rescue was about 75%. This result may be explained by the fact that only 30% of the cells were transfected at the passage used in this experiment (data not shown). Thus inhibition of the p38 MAP kinase pathway, either by the specific p38 inhibitor SB 202190 (Fig. 3C) or by the expression of p38 dn in NWTb3 cells (Fig. 5),
TABLE I

DNA content analysis by FACS, cell cycle arrest by 4-NQO and rescue by IGF-I

Numbers in parentheses represent a control experiment with no 4-NQO treatment (n = 2).

| Time | IGF-I | Cells in cell cycle | % | S | G2–M |
|------|-------|---------------------|----|---|------|
| 0    | -     | 66                  | 15 | 19|      |
| +    | 65    | 17                  | 18 |    |      |
| 2    | -     | 79 (59)             | 9  | 12| 18 (23)|
| +    | 69 (54)| 15                  | 20 | 16| (26) |
| 4    | -     | 85 (72)             | 6  | 19| (18) |
| +    | 61 (68)| 12                  | 16| (20)|      |

* The time after 2.5 μM 4-NQO treatment (30 min at 37 °C) of NWTb3 cells is indicated in days.

** Past-treatment with 5 nM IGF-I.

Fig. 2. Effect of the IGF-IR activation on 4-NQO-induced cell DNA damage. NWTb3 cells were plated in DMEM, 10% FBS in 6-well plates. After 15 h, the medium was changed to DMEM, 1% FBS for overnight starvation. Cells were untreated or treated with 2.5 μM 4-NQO for 30 min at 37 °C. The drug was removed by washing the cells with medium, and cells were incubated for the indicated time in DMEM, 5% FBS with or without 50 nM IGF-I. At each day of the experiment, cells were scraped gently and counted. 1 x 10^6 cells/ml were used for the comet assay (single cell gel electrophoresis) that was performed as described under “Experimental Procedures.” Slides containing cells were viewed by fluorescence microscopy; 200 cells were counted, and DNA-damaged cells were recognized by their comet (tail DNA). The percentage of comet cells for 200 total cells is represented on the figure that shows a representative experiment of comet assay (n = 2). The figure legend is as follows: +C, negative control (no treatment); +C, positive control (cell treatment 100 μM H2O2 for 20 min at 4 °C); +4-NQO, treatment with 2.5 μM 4-NQO; +IGF-I, incubation with 50 nM IGF-I. Days 4 and 5 indicate the time after 4-NQO treatment when the assay was performed.

results in a marked reduction of the IGF-I-induced rescue of cells treated with 4-NQO.

HCR of β-Galactosidase Activity for the UV DNA-damaged Adenovirus Is Enhanced by IGF-I—In order to determine the role of IGF-I in DNA repair, we employed an adenoviral vector that readily infects cells. The recombinant adenoviral vector AdCA35lacZ contains the β-galactosidase reporter gene under the control of the MCMV immediate early promoter inserted into the deleted E1 region of the virus (15). This vector can efficiently infect and express the reporter gene in cells but is unable to replicate in the absence of exogenous E1 expression. We damaged the adenovirus DNA using UVC irradiation (wavelength 354 nm), and we determined whether IGF-I was able to induce repair of this DNA-damaged adenovirus. The experiment consisted of irradiating the adenovirus DNA with UVC, infecting NWTb3 cells with damaged or undamaged adenovirus, and incubating these cells for 48 h with or without IGF-I. Thus, transcription of the β-galactosidase reporter gene present in the virus genome serves as an indicator of DNA repair after UV damage.

Since it has been suggested that increased survival (or enhanced reactivation) of DNA-damaged viruses results from an induced DNA repair pathway, we first examined the survival of AdCA35lacZ in NWTb3 cells. A typical survival curve (host cell reactivation or HCR) of β-galactosidase activity for the UV-irradiated AdCA35lacZ in NWTb3 cells is represented in Fig. 6A. Cells were infected at an m.o.i. of 40 pfu/cell with the AdCA35lacZ that was irradiated with UV doses ranging from 0 to 1000 J/m^2. Fig. 6A shows that the decrease in reporter gene activity is related to the degree of UV exposure of the adenovirus used to infect NWTb3 cells.

In order to determine whether IGF-I affects DNA repair, we examined its effect on the HCR of β-galactosidase activity for the UV-damaged AdCA35lacZ. We infected NWTb3 cells with non-irradiated AdCA35lacZ or virus that had been UV-irradiated over a dosage range encompassing the IC50 (Fig. 6A, 6B, 300, 500, and 800 J/m^2). After infection, cells were incubated for 48 h in the absence or in the presence of 50 nM IGF-I in the medium. Fig. 6B shows the resulting relative β-galactosidase activity and represents the DNA repair of the UV-damaged reporter gene. As shown in the figure, IGF-I is able to enhance the relative β-galactosidase expression from the damaged adenovirus. This result represents significant DNA repair induced by the addition of IGF-I.

DISCUSSION

IGF-I is a major survival factor, protecting cells from apoptosis induced by a variety of agents such as growth factor withdrawal (22) and etoposide ADDIN ENRfu (23). In the work reported here, we were interested in studying the ability of IGF-I, through stimulation of its receptor, to rescue cells from UV-like DNA damage. Toward this end, we used 4-NQO as an UV-mimetic agent to damage mouse fibroblast cells. This agent is one of the few chemical carcinogens whose metabolic processes are well characterized (24). Treatment with 4-NQO has been employed in mammalian cells as a paradigm for DNA damage-induced carcinogenesis. To exert its neoplastic effect, 4-NQO must undergo metabolic activation to the proximate carcinogen 4-hydroxyaminoquinoline 1-oxide, which, after acylation, reacts with DNA to form stable quinoline-purine monoadducts, i.e. at the exocyclic N-2 and N-6 positions of guanine and adenine, respectively (25). 4-NQO has been characterized as “UV-mimetic” with respect to its genotoxic properties; it behaves very similar to UVC light (and ionizing radiation) in several DNA damages systems (26). Both bulky 4-NQO adducts and UV light-induced pyrimidine dimers can be repaired by nucleotide excision repair pathways (27).

In this study, we used NWTb3 cells, a NIH-3T3-derived cell line overexpressing the normal human IGF-IR (13). For control cells, we also used tyrosine kinase-deficient human IGF-I receptors where the lysine 1003 residue at the ATP-binding site was substituted by alanine (KA mutant) or arginine (KR mutant) (14). The resulting cell lines overexpressing these constructs were characterized as dominant negative NIH-3T3-derived cell lines, named NKA8 and NKR1, respectively. Both were used for comparison with NWTb3. By using a cellular proliferation assay, we demonstrated that the IGF-IR activation was capable of rescuing NWTb3 cells from DNA damage induced by 4-NQO. The kinase activity of IGF-I is essential for the rescue of cells from DNA damage since no rescue occurred with NKA8 or NKR1 damaged using 4-NQO. These mutated IGF-IRs function in a classic dominant negative man-
ner, and it has been shown that addition of IGF-I to cells expressing these mutant IGF-IRs fails to protect cells from apoptosis (5). In a separate study, Kuhn et al. (12) demonstrated that the activation of IGF-IR promotes survival of human keratinocytes following UVB irradiation. These cells are exquisitely more susceptible to UVB-induced apoptosis if the growth medium is depleted of exogenous growth factors. Furthermore, we demonstrate here that IGF-I was also able to act directly on DNA damage caused by 4-NQO, since we determined that IGF-I induced a decrease in cell DNA damage as demonstrated by the comet assay.

It has been shown that protection of cells by IGF-I against apoptosis is commonly mediated by activation of multiple signal transduction pathways including PI3K and MAPK cas-
The adenovirus was previously damaged by various doses of UV irradiation as shown on the figure. Cells were harvested 48 h after infection, and (3 replicates) were combined and represented as the mean.

The finding that IGF-I-induced activation of p38 can be inhibited by SB 202190 suggests the activation of p38 MAPK by IGF-I, since p38γ and -δ are not inhibited by SB 202190 (33). Further experiments will be necessary to address the activation state of p38γ and -δ in IGF-I-stimulated cells since it has been shown that activation of p38γ, but not the other isoforms, is required for γ-irradiation-induced G2 arrest (34). Thus in certain cells, p38γ and -δ may mediate cell cycle arrest, whereas α and β isoforms may mediate rescue from DNA damage and cellular proliferation.

Previous studies performed by McKay et al. (35) have used-enhanced apoptotic death of differentiating neurons. The p38α/MEF2 pathway seemed to protect differentiating cells from death during neurogenesis. Moreover, Scrimgeour et al. (29) showed that neither MAPK nor PI3K activation is entirely sufficient for the full mitogenic/tumorigenic effect of the IGF-IR in NIH-3T3 cells. Thus there must be additional signaling pathways emanating from IGF-IR in addition to the well-described pathways.

Consideration of the results obtained when NWTb3 cells were transfected with a dominant negative p38 MAP kinase (pCMV5-p38AGF) suggests that the p38 pathway plays a role in the IGF-IR-mediated rescue of cells from DNA damage. Although activation of p38 MAPK was shown to play a role in apoptosis in the PC12 neuronal cell line (30), other recent studies have reported the anti-apoptotic effect of p38 MAPK in some cell lines and the role of p38 MAPK in cell proliferation. For instance, it has been shown that expression of wild type MKK4 and MKK6 that phosphorylate and activate p38 enhanced cell survival in the presence of tumor necrosis factor-α (31). Moreover, by using specific chemical inhibitors of MAP kinases, Rausch and Marshall (32) demonstrated that both ERK and p38 pathways are critically involved in the transduction of a proliferative signal and cooperate in granulocyte-colony-stimulating factor (G-CSF)-induced proliferation.

In this study, we found that inhibition of p38 MAPK by SB 202190 blocks the cell proliferation rescue induced by IGF-I. The ability of IGF-I to rescue cells from DNA damage is primarily blocked by SB 202190, a specific inhibitor of p38 MAPK activity, whereas LY 294002 (PI3K inhibitor) and U 0126 (ERK1/2 inhibitor) did not block the IGF-I-induced rescue. Thus the rescue pathway used by the IGF-I system apparently acts primarily through the p38 MAP kinase cascade. Recently, Okamato et al. (28) described that a dominant negative effect of p38 MAPK enhanced p38 MAPK-dependent apoptosis. Therefore, a dominant negative effect of p38 MAPK has been used to determine the role of p38 MAPK in the IGF-I-induced rescue of cells from DNA damage.

The viral DNA was previously damaged (or not) following UV irradiation at different doses (determined based on A). Thereafter, the medium containing excess virus was removed by washing the cells twice. Cells were then incubated in the absence (open bars) or in the presence of 50 nM IGF-I in DMEM with 1% FBS. Cells were harvested 48 h after infection, and the β-galactosidase activity was assayed as described under “Experimental Procedures.” Each point is the average of 3 replicates ± S.E.

In this study, we found that inhibition of p38 MAPK by SB 202190 blocks the cell proliferation rescue induced by IGF-I. The finding that IGF-I-induced activation of p38 can be inhibited by SB 202190 suggests the activation of p38α or β isoforms by IGF-I, since p38γ and -δ are not inhibited by SB 202190 (33). Further experiments will be necessary to address the activation state of p38γ and -δ in IGF-I-stimulated cells since it has been shown that activation of p38γ, but not the other isoforms, is required for γ-irradiation-induced G2 arrest (34). Thus in certain cells, p38γ and -δ may mediate cell cycle arrest, whereas α and β isoforms may mediate rescue from DNA damage and cellular proliferation.
an UV-damaged adenovirus as a tool to demonstrate the capacity of UV-irradiated cells to support viral DNA synthesis involving transcription-coupled repair of UV-damaged DNA. Similarly, a recombinant adenovirus, Ad5HMCVsp1lacZ, has been used to study the effect of heat shock on DNA repair of an UV-damaged reporter gene (36), and an adenoviral vector has been also used to prove the existence of an UV-inducible error-prone DNA repair mechanism in normal cells (19). Thus, host cell reactivation of UV-damaged nuclear replicating viruses has been used to assess the DNA repair ability of a variety of cell types. In the present study, we determined rescue of viral DNA damage induced by IGF-I in normal fibroblasts. Toward this end, we employed AdCA35lacZ and measured the DNA repair capacity of NWTb3 cells through the host cell reactivation technique. NIH-3T3-derived fibroblasts were infected with AdCA35lacZ in which the reporter gene expression was under the control of an MCMV immediate early promoter, a promoter known to drive high levels of expression without any species preferences as has been observed for the human cytomegalovirus promoter (15). We demonstrate here that IGF-IR activation is able to facilitate repair of exogenous damaged DNA. The data show the sensitivity of exogenous DNA repair to IGF-IR activation and confirm the utility and convenience of using adenoviral vectors to study this process (35, 37). Indeed, this latter experimental approach may be of particular value in order to examine repair of DNA damaged by a variety of different physical and chemical agents.

In summary, our results indicate that IGF-I is able to rescue cells from DNA damage through activation of its receptor primarily via the p38 MAP kinase signaling pathway. We additionally demonstrate IGF-I-specific induction of DNA repair on exogenous (adenoviral) UV-damaged DNA. This adenoviral technique may be of importance in extending DNA repair studies in order to examine the repair of DNA damaged by a variety of different physical and chemical agents. IGF-I is a powerful anti-apoptotic agent, and the IGF-IR activation may be important in inducing DNA repair and allowing the cell cycle to progress following DNA damage.

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REFERENCES

1. Butler, A. A., Yakar, S., Gewolb, I. H., Karas, M., Okubo, Y., and LeRoith, D. (1998) Comp. Biochem. Physiol. B Biochem. Mol. Biol. 121, 19–26
2. LeRoith, D., Werner, H., Beitiner-Johnson, D., and Roberts, C. T., Jr. (1995) Endocr. Rev. 16, 143–163
3. Baserga, R., Hongo, A., Ruini, M., Prisco, M., and Valentinis, B. (1997) Biochem. Biophys. Res. Acta 1332, F105–F126
4. Qu, B. H., Karas, M., Koval, A., and LeRoith, D. (1999) J. Biol. Chem. 274, 31179–31184
5. Kellie, O. J., Klippel, A., and Weber, M. J. (1997) Mol. Cell. Biol. 17, 1595–1606
6. Kulik, G., and Weber, M. J. (1998) Mol. Cell. Biol. 18, 6711–6718
7. Parrizas, M., Saltiel, A. R., and LeRoith, D. (1997) J. Biol. Chem. 272, 154–161
8. Okajima, Y., Matsunuma, I., Nishiu, T., Hashimoto, K., Yoshida, H., Ishikawa, J., Wakas, H., Yoshimura, N., Kanzakura, Y., Tomiyama, Y., and Matsuzawa, Y. (1998) J. Biol. Chem. 273, 22877–22883
9. Beck, K. D. (1994) Prog. Neurobiol. (New York) 44, 497–516
10. Pugazhenthi, S., Boras, T., O’Connor, D., Meintzer, M. K., Heidenreich, K. A., and Reusch, J. E. (1999) J. Biol. Chem. 274, 2929–2937
11. Heck, S., Lezoualc’h, F., Engert, S., and Behl, C. (1999) J. Biol. Chem. 274, 9828–9835
12. Kuhn, C., Hurwitz, S. A., Kumar, M. G., Cotton, J., and Spandau, D. F. (1999) Int. J. Cancer 80, 431–438
13. Blakesley, V. A., Kato, H., Roberts, C. T., Jr., and LeRoith, D. (1995) J. Biol. Chem. 270, 2764–2769
14. Kato, H., Faria, T. N., Stannard, B., Roberts, C. T., Jr., and LeRoith, D. (1993) J. Biol. Chem. 268, 2655–2661
15. Addison, C. L., Hitt, M., Kunskens, D., and Graham, F. L. (1997) J. Gen. Virol. 78, 1653–1661
16. Raines, J., Gupta, S., Rogers, J. S., Dickens, M., Han, J., Ulevitch, R. J., and Davis, R. J. (1995) J. Biol. Chem. 270, 7420–7426
17. Ostling, O., and Johanson, K. J. (1984) Biochem. Biophys. Res. Commun. 123, 291–298
18. Graham, F. L., and Eb, A. J. V. D. (1973) J. Virol. 52, 456–467
19. Bennett, C. B., and Rainbow, A. J. (1988) Mutagenesis 3, 157–164
20. Blakesley, V. A., Koval, A. P., Stannard, B. S., Sringle, G., and LeRoith, D. (1998) J. Biol. Chem. 273, 18411–18422
21. Lee, J. C., and Young, P. R. (1996) J. Biol. Chem. 271, 535–540
22. Rodriguez-Tarduchy, G., Collins, M. K., Garcia, I., and Lopez-Rivas, A. (1992) J. Immunol. 149, 535–540
23. Sell, C., Baserga, R., and Robin, R. (1995) Cancer Res. 55, 303–306
24. Kiyohara, C., Hirohata, T., Kuratsune, M., and Nagayama, J. (1991) Mutat. Res. 246, 111–117
25. Tada, M. (1975) Nature 255, 510–512
26. Mirzayans, R., Smith, B. P., and Paterson, M. C. (1989) Cancer Res. 49, 5523–5529
27. Snyderwine, E. G., and Bohr, V. A. (1992) Cancer Res. 52, 4183–4189
28. Okamoto, S., Kraine, B., Sherman, K., and Lipton, S. A. (2000) Proc. Natl. Acad. Sci. U. S. A. 97, 7561–7566
29. Sringle, A. G., Blakesley, V. A., Stannard, B. S., and LeRoith, D. (1997) Endocrinology 138, 2552–2558
30. Xia, L., Dickens, M., Raines, J., Davis, R. J., and Greenberg, M. E. (1995) Science 270, 1326–1331
31. Roublon, A., Reinhard, C., Amiri, P., and Williams, L. T. (1998) J. Biol. Chem. 273, 10222–10229
32. Rausch, O., and Marshall, C. J. (1999) J. Biol. Chem. 274, 4096–4105
33. Nemoto, S., Xiang, J., Huang, S., and Lin, A. (1998) J. Biol. Chem. 273, 16415–16420
34. Wang, X., McGowan, C. H., Zhao, M., He, L., Downey, J. S., Fears, C., Wang, Y., Huang, S., and Han, J. (2000) Mol. Cell. Biol. 20, 4543–4552
35. McKay, B. C., Winrow, C., and Reusch, J. F. (1997) Photochem. Photobiol. 66, 659–664
36. McKay, B. C., and Rainbow, A. J. (1996) Mutat. Res. 363, 125–135
37. Delporte, C., Gabor, I. A. T., and LeRoith, D. (1998) Biochem. Biophys. Res. Commun. 246, 584–588