EXPERIMENTAL STUDY

Growth hormone protects against radiotherapy-induced cell death

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Abstract

Background: *In vivo* treatment with growth hormone reduces radiation-associated mortality. The molecular mechanisms underlying this effect are unknown. It has been described that increased sensitivity to ionising radiation can be due to defects in machinery involved in detection and/or repair of DNA double-strand breaks.

Objective: To study the mechanisms involved in growth hormone action on the increased survival in irradiated cells.

Materials and methods: CHO-4 cells stably expressing the growth hormone receptor were used. A cell viability assay was carried out to analyse the increase in survival induced by growth hormone in irradiated cells. To investigate whether the DNA repair mechanism could be implicated in this effect we performed DNA reactivation assays using pHIV-LUC and pCMV-βgal plasmids as control. Identical studies were also conducted using the radiomimetic drug, bleomycin.

Results: Growth hormone protects CHO-4 cells from bleomycin- and radiation-induced cell death. In pHIV-LUC transfected cells, a time-dependent decrease in luciferase activity was observed after irradiation in the absence of growth hormone. However, cells pretreated with this hormone maintained reporter activity. When cells were transfected with irradiated pHIV-LUC plasmid, only the hormone-treated cells recovered the transcriptional activity.

Conclusions: Growth hormone exerts a radioprotective effect in CHO-4 cells stably transfected with the complementary DNA for the rat growth hormone receptor. The radioprotection is triggered directly by the hormone and it is also observed with bleomycin. The increased survival in response to radiation and bleomycin treatment induced by growth hormone correlates with an enhanced ability of the cells to repair damaged DNA.

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Introduction

The cell nucleus is considered the primary target for the lethal effects of ionising radiation and the heterologous DNA double-strand breaks are the most common type of radiation lesions that lead to mammalian cell death (1). However, the outcome of this damage is not necessarily cell death since mammalian cells can repair radiation-induced DNA breaks proficiently (2). Radiation-induced damage in DNA and other cellular components triggers cascades of regulatory events in eukaryotic cells. These events constitute a complex network of pathways towards cell cycle checkpoints, DNA repair and damage tolerance mechanisms, recombination and programmed cell death (apoptosis). Several pieces of evidence suggest that certain growth factors and cytokines can modify cellular

radiosensitivity. Thus, basic fibroblast growth factor (bFGF) protects bovine aortic and murine intestinal endothelial cells against radiation-induced programmed cell death (3, 4); insulin-like growth factor (IGF)-I (5), keratinocyte growth factor (6), vascular endothelial growth factor (VEGF) (7) and acid and basic FGFs (8) exert protective effects on intestine from radiation injury; interleukin (IL)-1, IL-11, IL-12 and tumour necrosis factor (TNF)-α are radioprotective in the murine bone marrow (9, 10, 11). Given before radiation, IL-1, IL-11 and transforming growth factor (TGF-β)-increase the number of surviving intestinal crypts after radiation (12–14).

Growth hormone (GH) is an anabolic hormone with pleiotropic effects on growth, differentiation and metabolism of cells (15), although it does not stimulate *in vitro* human tumour growth (15). In general, GH

initiates its biological actions by interaction with a specific membrane-bound receptor (15). The GH receptor is phosphorylated upon ligand stimulation by physical association with the non-receptor tyrosine kinase JAK2. JAK kinases are linked to transcriptional regulation and their activation results in the phosphorylation, dimerisation, and nuclear translocation of latent cytoplasmic STAT transcription factors. These activated STAT factors bind to their appropriate DNA-responsive elements and activate gene transcription (16).

GH has been clinically used in GH-deficient children (17) and adults (18), in patients recovering from surgery (19) and in patients with intestinal diseases like Crohn's syndrome (20) and inflammatory bowel disease (21). GH stimulates muscle protein synthesis, improves the nitrogen balance and promotes wound healing in a variety of catabolic states (22, 23). In addition, GH has been shown to reduce radiation-associated mortality (24). In this context, it has been reported that, within the bowel of radiated rats, GH favours the absorptive process by increasing mucosal proliferation and villous size (24). This protection exhibits an increase in intestinal crypt proliferation and a reduction in apoptosis. The mechanism by which GH reduces the effect of radiation is unknown. Here we used an *in vitro* cell system to further investigate the influence of GH on radiation responses. The results presented here indicate that GH has a protective action against radiation and this effect seems to be mediated by its ability to increase the repair of radiation damaged DNA.

Materials and methods

Cell lines and plasmids

Chinese hamster ovary (CHO)-4 and human embryonic kidney (HEK) 293T cells were cultured in Ham's F-12 medium or Dulbecco's modified Eagle's medium respectively containing 4.5 g/l glucose and supplemented with 10% fetal calf serum (FCS) and 1 mM L-glutamine. pHIV-LUC (25) contains sequences -453/+80 from the HIV enhancer linked to the firefly luciferase gene. PCMV- β gal plasmid contains the prokaryotic β -galactosidase gene inserted between the cytomegalovirus promoter (CMV) and the SV40 splice and polyadenylation signals. SPIGLE1 plasmid containing the GAS sequence from the SPI 2.1 promoter was kindly provided by Dr Ugo Moens (26).

DNA reactivation assays

CHO-4 cells were plated 24 h before transfection at a density of 3×10^5 cells/60 mm plate. Cells were transfected with the plasmid pHIV-LUC by the calcium phosphate method as described (25). Sixteen hours after transfection cells were fed with low serum

medium (0.5%) and treated with different concentrations of recombinant human GH (hGH, Serono, Madrid, Spain) for a further 4 h. Then, cells were irradiated using a telecobaltotherapy unit (60CO27 Model Theratron-80, MDS Nordion, Kanata, Ontario, Canada). Cell extracts were obtained as described before (25) at different times after irradiation and assayed for luciferase activity. Alternatively pHIV-LUC plasmid was irradiated with different doses and transfected together with pCMV- β gal plasmid as a control, by the calcium phosphate method. Sixteen hours after medium was removed, cells were fed with low serum medium (0.5%) and supplemented when indicated with different doses of GH. Cells extracts were prepared 48 h after transfection and assayed for β -galactosidase and luciferase activity. Fold activation was obtained by normalising luciferase activity to β -galactosidase expression.

Gene expression analysis

Cells were transfected with the indicated plasmids and harvested at different times after treatment. Protein extracts were prepared by three consecutive cycles of freezing and thawing. The total amount of protein was determined with a commercial kit based on the Bradford method (BioRad, Hemel Hempstead, UK). Ten to twenty micrograms of protein was assayed for cloramphenicol acetyl transferase (CAT) activity using a xylene-based method. Briefly, total volume for each cellular extract was adjusted to 85 μ l with 0.25 M Tris-HCl (pH 7.5) and then a mixture containing 32 μ l 0.25 M Tris-HCl, 5 μ l butyryl CoA (5 mg/ml) and 3 μ l [14 C]cloramphenicol (0.1 mCi/ml) was added. The reaction mixture was incubated for 4 h at 37°C and the reaction was stopped by addition of a mixture of 2:1 2,6,10,14-tetramethylpentadecane (Pristane, Sigma): xylene isomer (Sigma, St Louis, Missouri, USA). The mixture was shaken vigorously for 30 s and spun at 14000 r.p.m. for 15 min. Two hundred microlitres of the upper phase were taken and added to 4 ml Optiphase Highsafe 2 liquid scintillation cocktail (Perkin Elmer Life Sciences, Boston, USA). Total counts (counts per minute, c.p.m.) were detected using a 1214 beta liquid scintillation counter and normalised against micrograms of protein. Luciferase activity was determined with a commercial kit (Promega, Madison, WI, USA). β -galactosidase activity was assayed as described elsewhere (25) by using *o*-p-nitrophenil phosphate as a substrate.

Cell viability assay

Cell viability was studied using a Crystal Violet-based staining method. Briefly, cells were plated in 60 mm plates at a density of 1×10^4 cells/plate. Sixteen hours after seeding cells were serum depleted and treated with different doses of GH during a further 4 h and subjected to different doses of radiation. Cells

were allowed to grow for 10 days, fixed with glutaraldehyde and stained with Crystal Violet as previously described (27). Alternatively cells were seeded at a density of 20×10^4 cells in 24-well dishes and stained after 72 h of treatment.

Results

Treatment of cells with GH increases cell survival after irradiation

Several *in vitro* models have been developed to study regulation via the GH receptor (28, 29). For this study we chose CHO-4 cells, which express rat GH receptor cDNA, and as a control cell line, HEK293T cells that are not responsive to GH. In order to establish the response conditions of the two cell lines we carried out transfection experiments using a reporter plasmid, SPIGLE1, containing the GAS sequence from the SPI 2.1 promoter and a STAT5 binding site (30). Both HEK293T and CHO-4 cells were transfected with either the reporter plasmid or the empty vector. As expected, even at high doses of GH, HEK293T cells did not present variations in promoter-driven transcription (Fig. 1). On the contrary, in CHO-4 cells, there was a dose-dependent activation of the promoter in response to GH at doses that have not been described to be antagonistic (31). These results confirmed that the two systems were suitable for our purposes. In order to investigate the potential role of GH in radiological protection, HEK293T and CHO-4 cells were seeded at low density in 60 mm dishes, deprived of serum for 16 h and treated with different doses of GH for 4 h. Cells were then subjected to irradiation and allowed to grow for 10 days. As shown in Fig. 2, no difference in the survival profile of HEK293T cells was observed in either the presence or absence of GH. In contrast, CHO-4 cells showed increased survival when they had been exposed to GH prior to irradiation (Fig. 2). This

effect was dose dependent, indicating that it was mediated by the biological activity of the GH receptor. We have observed that higher doses (400 nmol/l) were toxic for cells (data not shown); consequently the dose range used was 50–200 nmol/l. We have also studied the effect of GH when cells were treated with the radiomimetic drug, bleomycin, and another drug, vincristin, which does not affect DNA in any way (Fig. 3). Our results showed that GH was also able to protect CHO-4 cells from bleomycin-induced cell death. In contrast, when cells were treated with the microtubule interfering agent vincristin, GH was not able to protect cells from the cytotoxic activity of the drug. These findings suggest that the protection induced by GH can be specific against agents that provoke DNA double-strand breaks.

Treatment of cells with GH facilitates repair of transfected DNA

Although the protective effect observed in cells treated with GH could be due to interference by the hormone in the response of the cells to irradiation at different levels, the above results strongly support the hypothesis that GH increases the activity of the DNA repair machinery. In order to test this possibility we performed different assays measuring reactivation of gene expression after irradiation in the presence of GH, as an indicator of DNA repair. CHO-4 cells were transfected with the plasmid pHIV-LUC, starved of serum for 16 h, treated with GH for 4 h and then irradiated and collected at different times. No effect of GH on basal transcription of the pHIV-LUC reporter was detected (data not shown), indicating that any change in the activity could be due to repair of the damaged plasmid. As shown in Fig. 4A, a time-dependent decrease in luciferase activity was observed, after irradiation, in the absence of GH. In contrast, cells pretreated with GH maintained luciferase activity during the 24 h period, indicating that repair

Figure 1 Induction of STAT5 dependent transcription by GH in CHO-4 cells. HEK293T and CHO-4 cells were transfected with 5 µg SPIGLE1 plasmid or empty vector. After 16 h of growth in low serum medium, cells were stimulated with the indicated doses of GH, harvested after 6 h of treatment and processed for CAT activity. Data represent mean and standard deviation of a single experiment performed in triplicate. The experiment was performed three times in triplicate and the results were similar.

Figure 2 CHO-4 and HEK293T cell survival to radiation. HEK293T and CHO cells were seeded in 60 mm dishes, treated with GH at the indicated concentrations and irradiated at doses ranging between 0 and 5000 rads. After 10 days cell survival was estimated by Crystal Violet stain. Data represent means and standard deviations of a single experiment performed in triplicate. The experiments were carried out three times in triplicate and similar results were obtained.

of the transcribed strand was more active in the presence of GH. To confirm this effect of GH, additional experiments were carried out in CHO-4 cells transfected with pHIV-LUC reporter and exposed to different doses of the radiomimetic drug bleomycin in the presence or the absence of GH. As observed in Fig. 4B, bleomycin treatment produced a time- and dose-dependent decrease in luciferase activity. However, reporter activity was maintained up to 40 h after the addition of the drug when CHO-4 cells were pretreated with GH. The effect of GH on the sustaining transcriptional activity in the CHO-4 cells that were irradiated or treated with bleomycin, might be the result of inhibited DNA degradation rather than increased DNA repair. To clarify this possibility we treated the pHIV-LUC reporter with two different radiation doses and then transfected the irradiated DNA together with non-irradiated pCMV- β gal, as a control for transfection efficiency. After transfection, cells were serum depleted for 16 h, stimulated with different doses of GH and

collected 40 h after treatment. The results showed that transcription of pHIV-LUC decreased in a dose-dependent fashion when DNA was irradiated (Fig. 5). When cells were transfected with irradiated DNA (1000 rads), the recovery of transcription was similar with the two GH doses used. However, the highest GH dose was more effective than the lower doses when the radiation dose was 3000 rads. Since the results were corrected for differences in transfection efficiency, these experiments strongly suggest that the action of GH on transcription reactivation was due to repair of the damaged pHIV-LUC reporters.

Discussion

This study demonstrates that GH is radioprotective in CHO-4 cells that express the GH receptor and the protective action of GH on radiation-induced injury is dose dependent. A similar conclusion was obtained

Figure 3 CHO-4 cells survival after vincristin and bleomycin treatment. CHO cells were seeded in 24-well dishes, and 16 h later were treated with vincristin or bleomycin at the indicated concentrations in the presence or absence of GH (200 nmol/l). After 72 h cell survival was estimated by Crystal Violet stain. The data represent means and standard deviations from a single experiment performed in triplicate. The experiments were repeated twice and similar results were obtained.

Figure 4 Reactivation of pHIV-LUC expression in CHO-4 cells after treatment with radiotherapy and bleomycin. (A) CHO-4 cells were transfected with pHIV-LUC reporter vector. After 16 h of low serum growth cells were treated with GH at the indicated doses and irradiated with 1000 rads. Cell lysates were prepared at the indicated times and assayed for luciferase activity. Data represent the means of three experiments performed in duplicate. (B) CHO-4 cells were transfected with pHIV-LUC reporter vector. After 16 h of low serum growth cells were treated with GH at the indicated doses and treated with the indicated doses of bleomycin. Cell lysates were prepared at the indicated times and assayed for luciferase activity. Data represent the means and standard deviations from three experiments performed in duplicate.

when the radiomimetic drug, bleomycin, was used. Bleomycin, an agent also used in cancer treatment, has to be activated to induce direct oxidative damage to DNA (32). The rescue from cell death by GH seems to be selective for agents that damage DNA, since cells treated with the microtubule disturbing agent, vincristin, did not show any difference when they were pretreated with GH. Goh *et al.* (33) reported that GH treatment reduces the apoptosis induced by colchicine in CHO-4 cells that express the GH receptor. These authors measured apoptosis at short time intervals after colchicine treatment. Since our experiments measured cell survival at 10 days after radiation exposure and 72 h after drug treatment, we believe our data measure the fraction of survival after treatment more accurately. Many actions of GH are mediated by IGF-I, and both factors share many overlapping effects in both physiological and cellular terms; IGF-I possesses antiapoptotic effects. It even up-regulates the level of bcl-xL gene product (34), a

member of the antiapoptotic Bcl-2 family. In our study, the survival effect observed in GH-treated CHO-4 cells was not due to induced IGF-I secretion, since CHO-4 cells neither synthesise nor secrete IGF-I either when deprived of serum or after GH stimulation (35, 31).

One of the mechanisms for radioprotection involves induction of biochemical and enzymatic pathways that prevent radiation-induced damage and/or promote DNA repair (36). We have found that plasmid DNA transfected in CHO-4 cells was more actively transcribed in cells treated with GH than in non-treated cells. Transcription activity also recovered more quickly in GH-treated cells exposed to bleomycin. These results suggest that GH activates pathways involved in DNA repair processes. Another explanation for our results would be that cell death inhibition, induced by GH, could protect either plasmid DNA or the protein product from degradation, and this would produce a more active transcription. However, reporter plasmid transcription was also more active in cells transfected with radiation-damaged DNA when

Figure 5 Reactivation of irradiated pHIV-LUC expression in CHO-4 cells after treatment with GH. pHIV-LUC plasmid was treated with two doses of γ -radiation (1000 and 3000 rads) and 2 μ g were cotransfected with 5 μ g non-irradiated pCMV- β gal plasmid in CHO-4 cells. After 16 h of low serum growth cells were treated with GH. Cell lysates were prepared after 40 h and assayed for luciferase activity and β -galactosidase. Efficiency of transfection was corrected as indicated in Materials and methods. Data represent means and standard deviations from three experiments performed in duplicate.

they were pre-treated with GH. Since the cells themselves were not exposed to radiation, the observed effects cannot be a consequence of apoptotic protection and suggest that DNA repair has been activated. This finding is also consistent with a recent study in rat liver (37) showing that GH induces the expression of several genes implicated in the control of DNA damage and in the response of cells to stress. Namely, GADD45, which inhibits mitotic growth (38), and APEN, which is both a DNA repair enzyme (39) and an activator of several transcription factors (40). Thus, GH may have a role in defending against cell stress and DNA damage. In this line, the results shown here may also explain an effect reported by our group in rats which were exposed to abdominal γ -radiation and whose survival was increased by 50% if the animals had been treated with human GH (24). The increase in survival was associated with reduced bacterial translocation, speedier increases in mucosal thickness and proliferative index and a decrease in apoptosis, suggesting that giving GH to irradiated rats promoted the adaptive process of the intestine against acute radiation-related negative effects.

Interaction of GH with its receptor induces the activation of signalling cascades including the JAK2 tyrosine kinase (41), MAPK (42, 31), activation of insulin receptor substrate (IRS)-1 and IRS-2 (43, 44), Phosphatidylinositol 3-kinase (45), Src homologous and collagen-like protein, Grb2 (46), Protein kinase C and phospholipase A2 (16) among others. Some of these pathways, particularly those driven by JAK2 and MAPK, can activate transcription mediated by STAT3 and STAT5 as well as SRF and c-fos (47, 30, 48) and probably activate expression of genes like GADD45 and APEN, which are involved in radiation-induced DNA repair. Alternatively, other signal transduction pathways activated by GH could be at the translation level and would activate the assemblage of DNA repair complexes. These possibilities are the subjects of ongoing research by our group.

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