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Gadd153 restores resistance to radiation-induced apoptosis after thiol depletion

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Abstract

It has been demonstrated that depletion of cellular thiols in lymphoma cells leads to an increased susceptibility of the cells to radiation-induced apoptosis. To elucidate the responsible mechanisms we used cDNA array hybridization to screen for differences in gene expression induced by thiol depletion. Among other genes we found the transcription factor Gadd153 to be upregulated after exposing the cells to cystine/methionine free culture medium. To study the functional consequences of this upregulation, cells constitutively expressing high levels of Gadd153 were generated by transfection. We found that overexpression of Gadd153 slightly reduced the amount of radiation-induced apoptosis in cells kept in normal medium but very significantly reduced apoptosis (from 55% to 18%) in cells which were treated with cystine/methionine free culture medium. The observed protective effect of Gadd153 against radiation-induced apoptosis in thiol depleted lymphoma cells argues for an anti-apoptotic function of Gadd153 after perturbation of the cellular redox state.

Keywords: Gadd153; Redox; Radiation; Bcl2; Lymphoma

Apoptosis was found to be important in determining the radiation response of tumors [1] and normal tissues [2] alike. Modulation of radiation-induced apoptosis therefore offers a strategy to improve the outcome in clinical radiation oncology. For this approach an interesting in vitro system consisting of the mouse lymphoma cell lines LYas and LYar has been published [3]. The radiosensitive cell line LYas, which is also very sensitive to radiation-induced apoptosis, gradually becomes more radioresistant by losing its high sensitivity to radiation-induced apoptosis during in vitro culture [3]. It was shown that this radioresistant cell line LYar shows high expression levels of the anti-apoptotic protein Bcl2 [4]. Interestingly the resistance of the Bcl2 overexpressing cell line LYar to radiation-induced apoptosis could be reversed to levels found in the sensitive parental cell line LYas by depleting the cellular thiols [4]. One of the reasons for the observed sensitization of LYar cells to radiation-induced apoptosis was found in the alteration of the cellular redox state which was caused by the depletion of thiols [5].

To further investigate the mechanisms responsible for this sensitization we screened for differences in gene expression in LYar cells, which were induced by the depletion of cellular thiols through incubation of the cells in cm^- culture medium. Among the transcript upregulated we found the mRNA of Gadd153. Gadd153 (growth arrest and DNA damage) also known as CHOP (C/EBP homology protein) is a nuclear protein that dimerizes with transcription factors belonging to the C/EBP family [6]. Gadd153 can function either as an inhibitor of transcription, because Gadd153-C/EBP dimers do not bind to classical C/EBP binding sites [6], or as an enhancer of transcription, because Gadd153-C/EBP dimers bind to specific DNA sequences [7]. Initially Gadd153 was identified as a protein, which is rapidly upregulated after exposing cells to DNA damaging agents [8]. Subsequently Gadd153 was found to be also responsive to other forms of cellular stress like glucose deprivation [9], oxidative stress [10] or perturbations of the endoplasmic reticulum [11]. Regarding the function

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of Gadd153 protein there is a significant amount of evidence indicating a pro-apoptotic function in several cell systems (e.g. [12–14]). Despite its well-established pro-apoptotic function the information about downstream effectors, which finally modulate the apoptotic response according to Gadd153 protein levels, is very limited.

Because of the mentioned pro-apoptotic function of Gadd153 we speculated that the upregulation of this protein, which was induced by treating LYar cells with cm^- medium, might be responsible for the observed sensitization to radiation-induced apoptosis. To test this hypothesis we overexpressed Gadd153 in LYar cells and found that the protein exhibits an anti-apoptotic function by reversing the thiol dependent sensitization to radiation-induced apoptosis.

Materials and methods

Cell culture. LYar cells were cultured in RPMI1640 containing 10% fetal bovine serum, 100 U/ml penicillin, and 100 μg/ml streptomycin at 37 °C in a humidified mixture of 95% air 5% CO_2. To deplete cellular thiols LYar cells were incubated in cm^- RPMI1640 (Sigma) containing the supplements mentioned above for 1 h prior to irradiation. Irradiations were performed at room temperature with an RT 100 (Philips) X-ray machine at 100 kV using a 1.7 mm Al filter. The dose rate was around 1.3 Gy/min.

Detection of apoptosis. Nuclear DNA fragmentation was determined exactly as published [3]. Briefly, cells were radiolabeled with 1.2 kBq/ml [^{14}C]thymidine (NEN) for 2 days immediately prior to the experiments. Six hours after irradiation the cells were harvested and lysed, and the amounts of soluble and insoluble DNA were determined by liquid scintillation counting.

Flow cytometric determination of apoptosis was performed essentially as described [15]. Six hours after irradiation cells were fixed in 70% ethanol at 4 °C for at least 30 min. Thereafter the cells were stained in a solution containing 50 μg/ml propidium iodide, 50 μg/ml RNAse A, 100 μM EDTA, and 0.1% Triton-X100 in PBS for 1 h at room temperature. After staining the cells were analyzed with an EPICS-Elite (Coulter) flow cytometer.

Array hybridization. mRNA expression levels were screened using Atlas (Clontech) cDNA expression arrays using essentially the procedures proposed by the manufacturer. Briefly 2 μg poly(A)$^+$ RNA from normal and cm^- treated LYar cells was reverse transcribed and radiolabeled in the presence of [α-^{33}P]dATP. These probes were hybridized to the Atlas Nylon Arrays (Clonetech) as suggested by the manufacturer. Hybridization signals were detected and quantified with a Cyclone phosphoimager (Packard).

Cloning and transfection of Gadd153. Poly(A)$^+$ RNA from LYar cells was prepared using the Fast Track 2.0 mRNA isolation Kit (Invitrogen) according to the instructions given by the manufacturer. The mRNA was reverse transcribed using the Gadd153 specific primer AGCCGAGCCCTCTCCTGGTCTAC with the SuperScript preamplification system (Gibco). From this cDNA the coding sequence of Gadd153 was amplified by PCR (20 cycles 95 °C, 1 min; 60 °C, 1 min; and 72 °C, 2 min) using the primer mentioned for cDNA synthesis and the primer GAACCTGGTCCACGTGCAGTCAT. The 639 bp PCR product was cloned into the pCR2.1 vector using a TA cloning kit (Invitrogen). The identity of the cloned cDNA with the published sequence (GenBank Accession No. X67083) was verified by sequencing. Thereafter the Gadd153 cDNA was subcloned into the retroviral expression vector pLXIN (Clontech) using the EcoRI restriction sites. The pLXIN–Gadd153 construct was transfected into the packaging cell line PT67 by electroporation. LYar cells were infected with the viral particles by incubation in PT67 cell culture supernatant in the presence of 10.8 μg/ml hexadimethrine-bromide. Cells containing an integrated pLXIN–Gadd153 construct were selected with 200 μg/ml G418 (Gibco), cloned, and expanded.

Western blots. Total cellular proteins were solubilized by boiling the cells in sample buffer [16] and separated on polyacrylamide gels. Proteins were electrotransferred to a nitrocellulose membrane (Schleicher, Schuell). After the membrane was blocked with BLOTTO, it was incubated for 1 h with the primary antibody. After three 15 min washes in TBST the membrane was incubated for 1 h in a 1:5000 dilution of peroxidase-conjugated secondary antibody (Boehringer–Mannheim). After three final 15 min washes in TBST, the blot was developed using a chemoluminescence detection kit (Boehringer–Mannheim).

Results

Hybridization of ^{33}P-labeled cDNA obtained from LYar cells cultured in normal and in cm^- culture medium to Atlas nylon arrays (Clonetech) was used to screen for differences in gene expression induced by the depletion of cellular thiols. Three hours after incubation of LYar in cm^- cell culture medium 60 out of 1176 spotted cDNAs were found to be differentially regulated. Among these a significant upregulation of the Gadd153 hybridization signal could be observed in thiol depleted LYar cells, which is shown in Fig. 1A. The cm^-

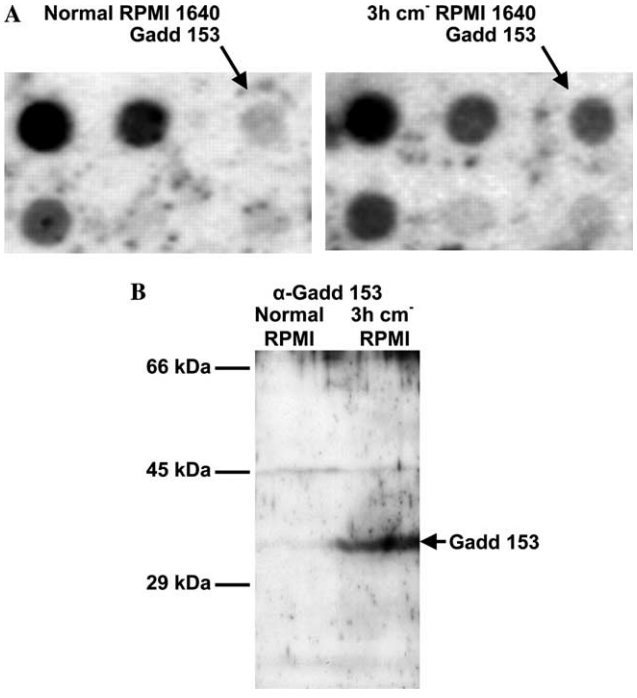

Fig. 1. Overexpression of Gadd153 in LYar cells after depleting cellular thiols by incubating the cells in cm^- culture medium. (A) Shows the result of the Atlas cDNA array (Clontech) hybridization indication an overexpression of Gadd153 mRNA 3 h after exposing the cells to cm^- medium. The Western blot in (B) demonstrates that the shown increase in Gadd153 mRNA leads to an increase in intracellular protein levels of Gadd153.

induced upregulation of the Gadd153 mRNA was verified by RT-PCR (results not shown) and the consecutive increase in the intracellular protein concentration could be demonstrated by immunoblot analysis, as shown in Fig. 1B.

To elucidate whether the observed upregulation of the transcription factor Gadd153 is responsible for the increased sensitivity of thiol depleted LYar cells to radiation-induced apoptosis, we permanently increased the level of Gadd153 protein by retroviral gene transfer. The amount of Gadd153 protein, which was expressed from the transgene under control of the LTR promoter in the pLXIN vector, can be seen on the Western blot in Fig. 2. Transfection of LYar cells with the pLXIN vector as well as with the pLXIN–Gadd153 construct did not change cell viability or cell cycle time significantly. We measured a time of 15 h for one population doubling in untransfected LYar cells while the doubling time was 17 h in pLXIN transfected and 16 h in Gadd153 overexpressing cells.

The effects of the forced expression of Gadd153 on radiation-induced apoptosis in LYar cells are shown in Fig. 3. The left panel of the diagram shows that LYar cells cultured in normal cell culture medium exhibit a DNA fragmentation of 5%. Six hours after irradiation with a dose of 2 Gy the level of apoptotic DNA fragmentation roughly doubles to 11%. An incubation of LYar cells in cm^- medium for 1 h prior to irradiation leads to a significant sensitization of the cells to radiation-induced apoptosis with a DNA fragmentation of

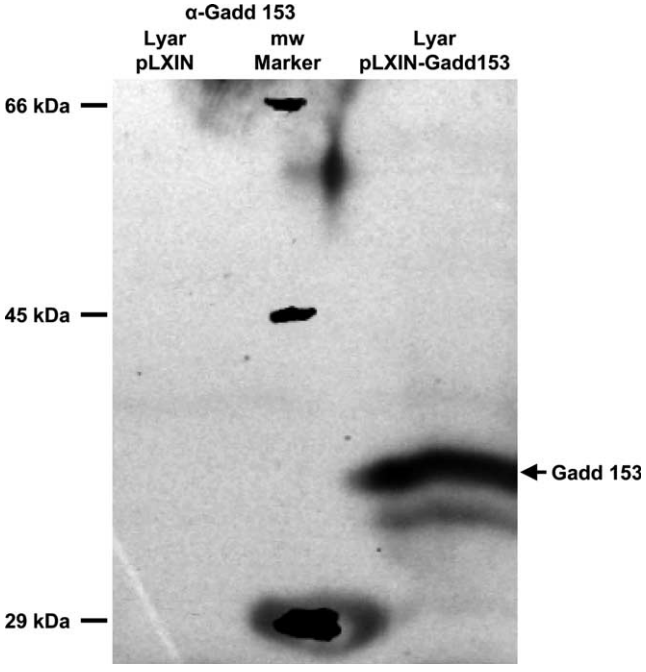

Fig. 2. Western blot depicting the overexpression of Gadd153 protein in cells transfected with pLXIN–Gadd153 (right lane) when compared to vector transfected control cells (left lane).

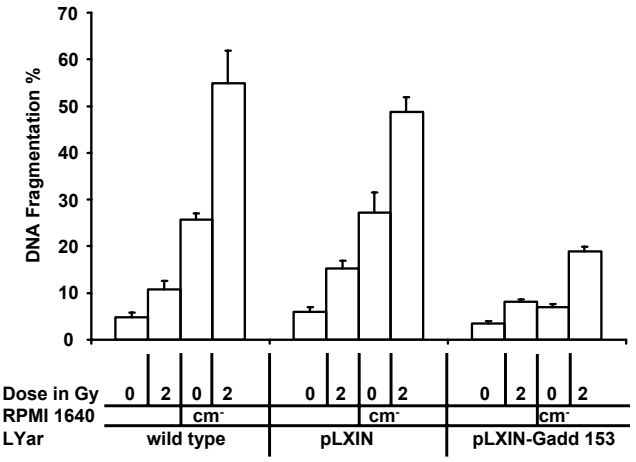

Fig. 3. Amount of radiation-induced apoptosis in untransfected LYar cells (wild type), vector transfected cells (pLXIN), and Gadd153 transfected cells (pLXIN–Gadd153). Apoptosis was determined 6 h after irradiation with 2 Gy. The error bars give the standard error of the mean of at least three independent experiments.

55% after exposure to 2 Gy. This observed sensitization of LYar cells to radiation-induced apoptosis by thiol depletion closely matches the data published by Mirkovic et al. [4]. The middle panel of Fig. 3 shows that the amount of background apoptosis as well as of radiation-induced apoptosis in cells, which are cultured either in normal RMPI1640 or in cm^- culture medium, did not change significantly when LYar cells were transfected with the vector pLXIN. The right panel shows the effects of the overexpression of Gadd153 protein. The amount of apoptosis found in untreated cells, which were cultured in normal RPMI1640, was 4% and therefore closely matched the amount found in wild type or vector transfected LYar cells. After irradiation with a dose of 2 Gy 8% of the transfected cells underwent apoptosis within 6 h. This fraction of apoptotic cells is again not very different to the fraction measured for untransfected cells. In contrast overexpression of Gadd153 leads to a drastically decreased apoptotic response in cells which were depleted of thiols by exposure to cm^- RPMI1640. Unirradiated LYar cells, overexpressing Gadd153, showed a DNA fragmentation of 7%, which is significantly ($p < 0.01$) lower than the DNA fragmentation rate of 27% which was determined for the vector transfected control cell line. Irradiation with a dose of 2 Gy induced an apoptotic response of 19% in Gadd153 overexpression cells, which were incubated in cm^- RPMI1640. This apoptotic response is significantly lower ($p < 0.01$) than the one observed in irradiated control or vector transfected LYar cells after thiol depletion. Therefore the overexpression of Gadd153 effectively reversed the sensitization of LYar cells against radiation-induced apoptosis by thiol depletion.

To support these findings obtained from the measurement of apoptotic DNA fragmentation DNA histograms were determined by flow cytometry. These are shown in Fig. 4. For the experiments shown in Fig. 4 LYar cells were transferred to cm$^-$ medium for 1 h and irradiated with the given dose, and the DNA histograms were measured 6 h after irradiation. The upper and the middle panels show the increase of apoptosis after irradiation as an increased sub-G_1-peak in normal and vector transfected cells. For both cell types the radiation-induced G_2 block is clearly visible. While the radiation-induced G_2 block is equally pronounced in irradiated cells and cells overexpressing Gadd153 (lower panel) the fraction of apoptotic sub G_1 cells is significantly reduced when compared to the control cells.

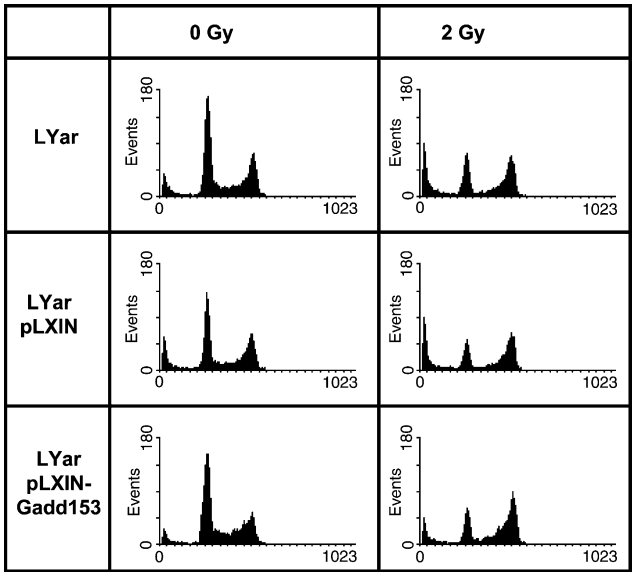

Fig. 4. Radiation-induced apoptosis in cm$^-$ treated LYar cells determined as sub G_1 peak by flow cytometry. Cells were fixed 6 h after irradiation. The upper panel shows DNA histograms of untransfected LYar cells, the middle panel of vector transfected cells, while the lower panel shows the DNA histograms of cells overexpressing Gadd153.

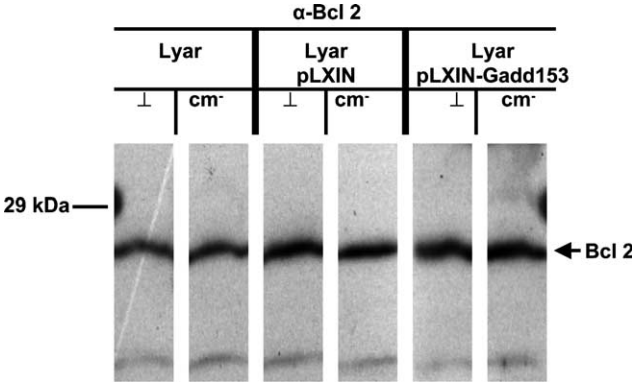

Fig. 5. Bcl2 proteins levels in normal as well as transfected LYar cells. The cells were lysed and analyzed by Western blotting 4 h after exposure to cm$^-$ culture medium.

It has been reported recently [17] that Gadd153 influences cell survival by the down-regulation of Bcl2. In addition literature [4] indicates that Bcl2 overexpression is a decisive step for the relatively low amount of radiation-induced apoptosis in LYar cells when compared to LYas cells. We therefore determined the intracellular Bcl2 concentration by immunoblotting as shown in Fig. 5. The blots shown in Fig. 5 demonstrate that Gadd153 overexpression did not change the level of Bcl2 in LYar cells. In addition the thiol depleting treatment with cm$^-$ RMPI1640 did not cause any change in the level of Bcl2 protein expression.

Discussion

It is well established that a number of cellular stresses like impaired protein folding in the endoplasmic reticulum [11], hypoxia [18], DNA damage [19], and oxidative stress [20] lead to an increase in the intracellular protein concentration of Gadd153. So the upregulation of Gadd153, which we observed after exposing LYar cells to cm$^-$ medium, can be readily explained as a reaction to the oxidative stress caused by the depletion of cellular thiols. This overexpression of Gadd153 could offer a plausible hypothesis for the increased sensitivity of cm$^-$ treated LYar cells to radiation-induced apoptosis if several published findings are considered:

- It was shown [12] that M1 myeloblastic leukemia cells, which overexpressed Gadd153 after the transfection of its cDNA, died through apoptosis.
- This publication [12] as well as [17] demonstrated that Gadd153 overexpression downregulated Bcl2, an anti-apoptotic protein, the overexpression of which has been implicated to be responsible for the low sensitivity of LYar cells to radiation-induced apoptosis [3].
- It has been demonstrated that forced overexpression of Gadd153 sensitizes the cell to substances like parthenolide [21], thapsigargin [17], or tunicamycin [17].

In sharp contrast to this hypothesis we found that forced overexpression of Gadd153 reduced the susceptibility of thiol depleted LYar cells to radiation-induced apoptosis. Gadd153 overexpressing cells, which were not treated with cm$^-$ medium, showed only a very modest reduction in the amount of radiation-induced apoptosis, a finding compatible with published findings that Gadd153 overexpression does not significantly alter the sensitivity of cells to ionizing radiation [17].

Despite the fact that our findings are contrary to the above-mentioned hypothesis there are reports in the literature which indicate an anti-apoptotic function of Gadd153 like we have seen in our experiments. In one report [22] it was demonstrated that fibroblasts which

show an increased resistance to oxidative stress also have elevated levels of Gadd153. More direct evidence for an anti-apoptotic function of Gadd153 comes from experiments with human mammary carcinoma cells, which were transfected with Gadd153 cDNA [23]. These experiments demonstrated that Gadd153 overexpression in itself as well as in combination with exogenous hGH significantly reduced the amount of apoptosis triggered by serum starvation of human mammary carcinoma cells.

Any effort to explain the observed antagonistic effect of Gadd153 overexpression on thiol depletion with regard to radiation-induced apoptosis is complicated by the small amount of information available about downstream effects of Gadd153 in the apoptotic cascade. Wang et al. [24] found three proteins which were upregulated in response to Gadd153 overexpression. One (DOC1) was found to be a novel form of carbonic anhydratase IV [25]. The second (DOC4) [24] was found to be homologous to a Drosophila protein known to play a role in embryonic development, while the third one (DOC6) [24] showed homologies to murine proteins binding to actin. None of these proteins have an established role in an apoptotic signal transduction cascade. Another downstream target of Gadd153, with a well-established function in apoptosis, was found to be glutathione (GSH) [17]. It was shown that Gadd153 overexpression reduced the intracellular GSH concentration at least twofold [17]. Considering our results one arrives at the conclusion that the interaction between Gadd153 protein levels and intracellular GSH concentration is considerably more complex than what previous publications [17] suggest.

We have shown that overexpressing Gadd153 counteracts the effect of GSH depletion with regard to radiation-induced apoptosis. Therefore if one looks at the modulation of cellular apoptosis by the interaction of Bcl2 and intracellular GSH concentrations (as reviewed in [26]) one also has to take the Gadd153 into account.

Acknowledgments

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