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Human growth hormone gene transfer into tumor cells may improve cancer chemotherapy

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Chemotherapy remains the main tool for the treatment of cancers, but is often hampered by tumor cell resistance. In this context, the transfer of genes able to accentuate the effect of anticancer drugs may constitute a useful approach, as exemplified by inactivation of nuclear factor (NF)-κB via direct transfer of a gene encoding a negative dominant of its natural inhibitor IκB, leading to improved response to cancer chemotherapy. Following our previous report that transfection of *human growth hormone* (*hGH*) gene into human monocytic cell lines may also inactivate NF-κB in another situation, we decided to test the consequences of *hGH* gene transfer on cancer treatments. We demonstrated that *hGH*-transfected human myeloid leukemia U937 cells were sensitized to an apoptotic signal mediated by the anticancer drugs. In parallel, we found that, by inhibiting degradation of IκB, *hGH* gene transfer diminished NF-κB entry into the nuclei of U937 cells exposed to daunorubicin. Finally, we report that *hGH*-transfected tumor cells engrafted in nude mice responded *in vivo* to chemotherapy with nontoxic doses of daunorubicin whereas, under the same conditions, control tumor cells remained insensitive. Overall, this study therefore suggests that *hGH* gene transfer may offer new therapeutic prospects in cancer therapy.
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Keywords: gene transfer; growth hormone; cancer; chemoresistance

Despite the introduction of new technologies for cancer treatment, the majority of tumors are still treated by chemotherapy. Nevertheless, tumor cells are often either refractory or poorly responsive to a broad range of anticancer drugs due to various mechanisms. Tumors may limit the effects of these drugs by causing their efflux through an active pump system encoded by multidrug resistance (MDR) genes (reviewed in Ref. [1]). Alternatively, because the effects of most anticancer agents are mediated via an active cell-death process, i.e., apoptosis,[2,3] tumor cells may react to overexpression of antiapoptotic oncoproteins or mutations of tumor suppressor genes.[4] Some authors have proposed to scale up the dosage of chemotherapeutic agents in patients with cancer to simply counteract chemoresistance. However, as an example, a 6-year survey of women treated with high-dose chemotherapy for advanced metastatic breast cancer showed no clear benefit for these patients[5] and severe toxic side effects. These poor results, therefore, encourage the search for new approaches to sensitize tumor cells to chemotherapy.

In this context, as the clinical use of drugs defined to reverse chemoresistance, such as chemical inhibitors of MDR genes, showed no potent effects,[6] gene delivery could be proposed as a way to potentiate the effect of anticancer drugs. One such approach consisted of transfer of the proapoptotic *bax* gene into ovarian cancer cell lines, which led to enhanced cytotoxicity in response to cisplatin or paclitaxel.[7] Several authors have also illustrated this concept by infecting tumor cells with an adenovirus encoding a negative dominant of IκB. The results clearly showed that the inhibition of nuclear factor (NF)-κB led to an increase in chemosensitivity of various cell lines in response to various drugs *in vitro*[8,9] and *in vivo*.[10,11]

In previous experiments, we demonstrated that *human growth hormone* (*hGH*) modulated the activation of NF-κB in response to bacterial lipopolysaccharides[12] and to TNF-α (manuscript in preparation). This prompted us to evaluate the *in vitro* and *in vivo* effect of *hGH* on other NF-κB stimulators, such as daunorubicin, an anthracycline widely used in cancer treatment. As reported in the present study, we found that *hGH* gene transfer sensitized human myeloid leukemia U937 cells to apoptosis mediated by daunorubicin. This phenomenon partly required activation of cysteine proteases called caspases responsible for cleavage of critical cell substrates, including poly-(ADP-ribose) polymerase (PARP) (reviewed in Ref. [13]). We also found that transfection of *hGH* led to a decrease in NF-κB translocation normally observed in response to daunorubicin, which could explain our observations. Finally, tumors obtained from *hGH*-transfected cells engrafted into nude mice clearly displayed

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a dose-dependent response to subtoxic doses of daunorubicin, whereas parental engrafted tumors remained insensitive to this treatment. We therefore propose a new method to potentiate the sensitivity of tumor cells to chemotherapy.

Methods

Cells and culture conditions

Cells used in this study, two different clones of the human myeloid leukemia U937 cells transfected with plasmid pLTR-hGH-SV5-Neo (U937-hGH clones) and one clone of U937 cells expressing Neo resistance only, as control cells (U937-Neo), have already been described.[14] Their relative growth rate was measured over 3-day cultures run in duplicate. They were maintained in RPMI 1640 (Bio-Whittaker, Verviers, Belgium) supplemented with 10% heat-inactivated FCS, 2 mM L-glutamine, 100 IU/mL penicillin, and 100 μg/mL streptomycin, under permanent selection in 0.5 mg/mL G418 (Life Technology, Paisley, Scotland).

Assessment of cell death

A total of 5×10^5 U937 cells in 0.5 mL of culture medium were cultured in the presence or absence of various quantities of daunorubicin (Cerubidin®, Roger Bellon Laboratory, Neuilly-sur-Seine, France). Hypoploid cell assessment was monitored on ethanol-permeabilized cells and stained using propidium iodide (PI; Sigma, St. Louis, MO) after an 18-hour culture, as previously described.[15] Cell death was detected by flow cytometry analysis (FACScan, Becton Dickinson, Mountain View, CA) of cell lines after a 48-hour culture, and stained for 15 minutes with 5 μg/mL PI and concomitantly 15 minutes at 37°C with 80 nM fluorochrome 3,3′ dihexyloxacarbocyanine iodide $DIOC_6(3)$ (Molecular Probes, Eugene, OR), allowing determination of the mitochondrial transmembrane potential ($\Delta\psi$m).[16] For each $\Delta\psi$m determination, quadrant gates were set to cell labeling with *m*-chlorophenylhydrazone (mClCCP, 15 minutes, 37°C). The concentration of daunorubicin allowing 50% inhibition of cell survival (IC_{50}) was measured in four separate experiments. The effect of Taxol® (kindly provided by Bristol-Meyers Squibb, Sermoneta, Italy) was assessed in other experiments under PI staining.

Lysates from 10^6 cells, with or without 24-hour exposure to daunorubicin, were separated on sodium dodecyl sulfate (SDS)–polyacrylamide gel and electroblotted onto PVDF membranes. Polyclonal rabbit Abs were used to recognize the 32-kDa pro–caspase-3 and the subunits of the active caspase-3 (Pharmingen, San Diego, CA). Cleavage of PARP was determined using the mouse C2-10 mAb (purchased from G Poirier, Montreal University, Canada). Blots were stained with either anti-rabbit peroxidase-labeled or anti-mouse peroxidase-labeled second Ab (Amersham, Amersham, UK) and were developed using an enhanced chemiluminescence detection system (ECL, Amersham), as previously reported.[14]

Determination of NF-κB activity

Cell lines were cultured for 3 hours in the presence of 5 μM daunorubicin. An electrophoretic mobility-shift assay was performed on equivalent quantities of nuclear extracts prepared from living cells. Specificity was assessed by incubating nuclear extracts obtained from daunorubicin-stimulated cells with nonradiolabeled NF-κB probe (5′-ACAAGGGACTTTCCGCTGGGGACTTTCCAG-3′) or mutated NF-κB oligonucleotide probe (5′-ACAACT-CACTTTCCGCTGCTCACTTTCCAG-3′), as previously reported.[12] The amount of NF-κB present in the gel was established by densitometric analysis of the bands (National Institutes of Health image software). The results are expressed in arbitrary units where 1 corresponds to the intensity of the untreated cell bands.

In separate experiments, 2.5×10^5 cells were cytospun and fixed in 3% paraformaldehyde and 0.025% glutaraldehyde at 4°C. Cells were then permeabilized with Triton X100 for 5 minutes, washed and stained with anti-p65 rabbit polyclonal Abs (Santa Cruz Biotechnology, Santa Cruz, CA), and revealed with fluorescein-labeled goat anti-rabbit polyclonal Abs. Cells were examined with an epifluorescent microscope (Leica, Jena, Germany).

Cell lysates obtained from 5×10^5 cells stimulated with 5 μM daunorubicin during 0, 30, 60, 90, and 120 minutes were separated on 10% SDS–polyacrylamide gel and electroblotted onto PVDF membranes. Blots were first stained with rabbit anti-IκBα Ab (Santa Cruz Biotechnology) and then with anti-rabbit peroxydase-labeled second Ab (Amersham). They were developed using an enhanced chemiluminescence detection system (ECL).

Determination of MDR activity measurement

A total of 5×10^5 U937-Neo or U937-hGH cells in 0.5 mL of regular culture medium were exposed to daunorubicin (5 μM) for 3 hours. These cells were then first incubated for 1 hour at 4°C with a mouse IgG2a anti-human P-glycoprotein (P-gp) mAb (Dako, Glostrup, Denmark) or with an irrelevant mouse IgG2a mAb (Dako), followed by a 30-minute incubation at 4°C with fluorescein-labeled goat anti-mouse Abs (Dako) and analyzed by cytofluorometry. In parallel, the doxorubicin-resistant MCF-7^{DXR} human breast cancer cell line,[17] maintained in a medium containing 10 μM doxorubicin, was prepared and analyzed under the same conditions.

In vivo *experiments*

Fragments (4 mm^3) from tumors obtained after subcutaneous injections of 5×10^6 U937-Neo and U937-hGH cells in irradiated nu/nu mice were engrafted into another series of mice. Treatment was started when tumors had developed to 60 to 300 mm^3. Mice were pooled and randomly assigned to three groups per tumor: mice not treated, mice receiving intraperitoneal (i.p.) injections of 0.75 mg/kg of daunorubicin and mice receiving i.p. injections of 1.5 mg/kg of daunorubicin. The daunorubicin dosage was determined in a preliminary study in groups of five non–tumor-bearing mice, showing that 1.5 mg/kg was the highest nontoxic dose. Animal body weights were recorded. Tumors were measured with a caliper by the same investigator and the volume was calculated according to the following

equation: $V\,(\mathrm{mm}^3)=d^2\,(\mathrm{mm}^2)\times D\,(\mathrm{mm})/2$, where d and D are the smallest and perpendicular diameters, respectively. Results were expressed as the relative tumoral volume (tumor volume at a particular day/tumor volume at day 0). For ethical reasons, animals were sacrificed under CO_2 anesthesia when tumor volume reached 2000 mm^3. Serum samples were collected at the time of sacrifice and circulating hGH was measured by ELISA (Life Technology, detection threshold of 500 pg/mL), as recommended by the manufacturer. All experiments were carried out under the conditions established by the European Union (Directive 86/609/EEC).

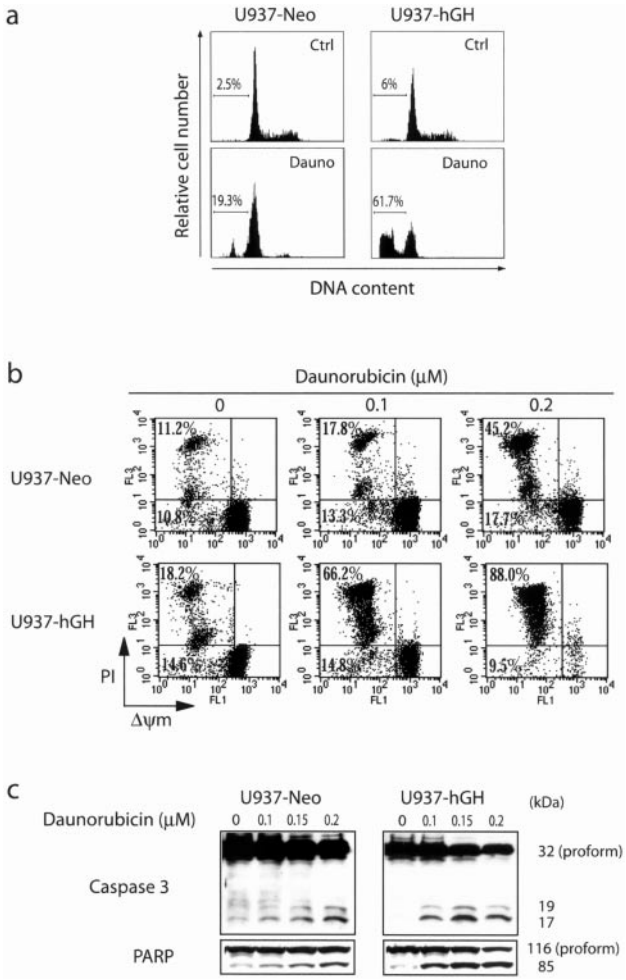

Figure 1 Effect of *hGH* gene transfer on daunorubicin-mediated apoptosis. U937-Neo and U937-hGH cell lines were cultured in the presence or absence of daunorubicin. **a:** The percentage of cells with hypodiploid DNA content was detected after staining ethanol-permeabilized cells with PI. Linear scales are represented. **b:** The percentage of dead cells was measured after staining with PI (upper left quadrant) and the percentage of dying cells was detected after labeling with the $DIOC_6(3)$ probe ($\Delta\psi$m, lower left quadrant). **c:** Nontreated cells or cells treated with various doses of daunorubicin were submitted to a 24-hour culture, before lysis for Western blot analysis. Caspase-3 activation was determined using anti–caspase-3 polyclonal Abs recognizing the pro–caspase-3 and its large subunits. Cleavage of PARP into an 85-kDa fragment was determined using the C2-10 mAb.

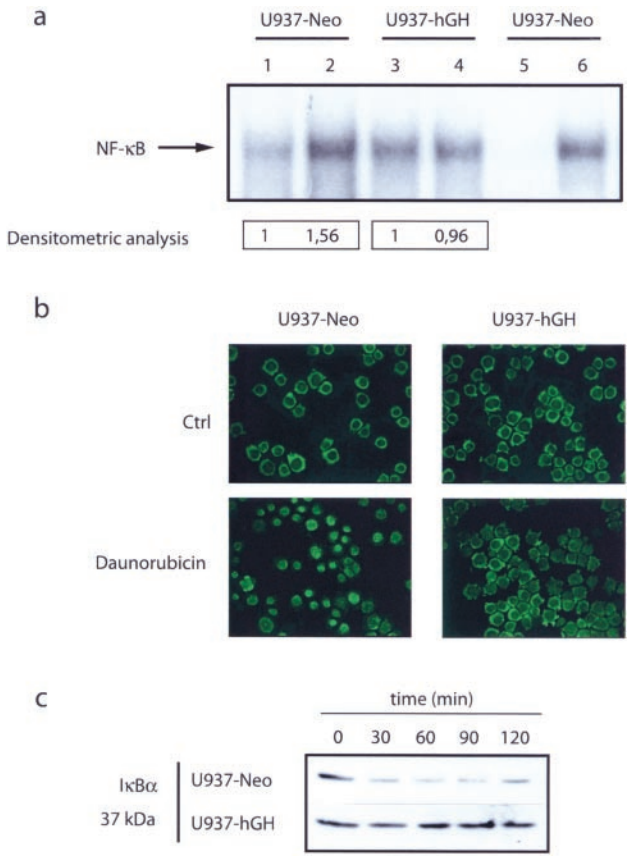

Figure 2 Effect of *hGH* gene transfer on NF-κB DNA binding activity following daunorubicin stimulation. **a:** An electrophoretic mobility-shift assay was performed on nuclear extracts as described in *Methods*. NF-κB migration was assessed by the migration of nuclear extracts from U937 cells stimulated with daunorubicin, coincubated with nonlabeled NF-κB–specific probe (lane 5) or nonlabeled NF-κB–mutated probe (lane 6). Control U937-Neo cells were either left unstimulated (lane 1) or stimulated with daunorubicin (lane 2). *hGH*-transfected cells were either left unstimulated (lane 3) or stimulated under the same conditions with daunorubicin (lane 4). Values correspond to the densitometric analysis of the gel bands. They are expressed in arbitrary units where 1 corresponds to the intensity of the untreated control cell bands. **b:** Cells cultured for 3 hours without or with 5 μM daunorubicin were treated as described in *Methods* before staining with anti-p65 polyclonal Abs. Cells were examined by epifluorescence microscopy. **c:** Whole cell extracts from our cell lines stimulated with daunorubicin for 0 to 120 minutes were subjected to Western blotting using anti-IκBα Abs under the conditions described in *Methods*.

Statistical analysis

Statistical analyses were performed using Student's *t* test for *in vitro* experiments and a nonparametric Kruskal-Wallis test for *in vivo* experiments.

Results

hGH gene transfer potentiates daunorubicin-induced apoptosis

The two different hGH-producing U937 cell lines (U937-hGH) and the U937-Neo control cell line were exposed to

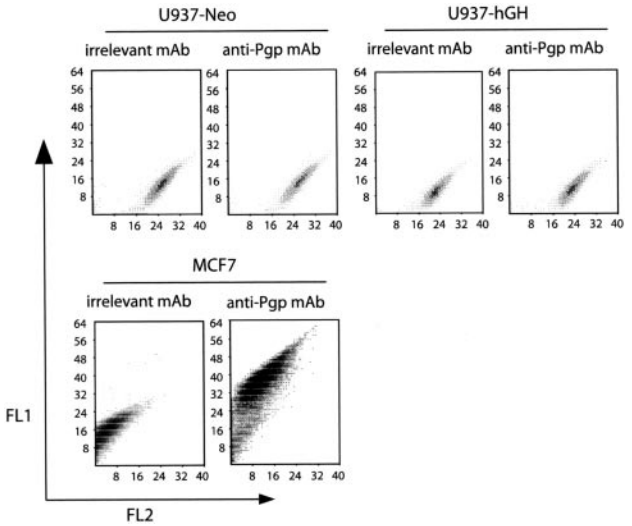

Figure 3 Effect of *hGH* gene transfer on modulation of MDR. U937-Neo and U937-hGH cells were stimulated with daunorubicin (5 μM) for 3 hours. Daunorubicin content was determined by measuring the mean fluorescent intensity in the FL2 channel, and membrane P-gp expression was determined by the differential shift observed in the FL1 channel when comparing cells labeled with anti–P-gp mAb to cells labeled with an irrelevant mAb. Doxorubicin-resistant MCF-7^{DXR} cells were used as positive controls.

daunorubicin. As shown in Figure 1a, cell death by apoptosis was evidenced by analysis of DNA content of permeabilized cells. Under these conditions, a marked increase in hypodiploid DNA was observed in daunorubicin-treated U937-hGH cells compared with untreated U937-hGH, whereas a slight difference was observed between untreated and treated U937-Neo cells. Cells were also stained with PI for detection of dead cells and with fluorochrome $DiOC_6(3)$ for determination of the mitochondrial transmembrane potential, another marker of apoptosis ($\Delta\psi$m).[16] As illustrated in Figure 1b, the percentages of apoptotic cells (PI+/$\Delta\psi m^{low}$) were higher for U937-hGH cells than for control U937-Neo cells, in response to increasing doses of daunorubicin. Based on the results of four independent experiments run with our two different hGH-producing cell lines, we obtained 62.27±2.45% of PI+U937-hGH cells treated with 0.1 μM daunorubicin, versus 14.16±1.26% PI+ treated parental cell lines (mean±SEM, P=.001). A separate study based on a wide range of daunorubicin concentrations (0.025 to 1 μM) indicated a 3.5 difference in IC_{50} (97.5 nM for U937-hGH cells vs 350 nM for U937-Neo cells). No significant difference was observed between untreated parental (9.48±1.50%, PI+ cells) and hGH-producing cell lines (12.52±1.20%, PI+ cells).

As reported in other cell lines,[18] daunorubicin treatment of U937-Neo cells induced activation of caspase-3 monitored by degradation of the 32-kDa pro–caspase-3 fragment into 20-, 19-, and 17-kDa fragments, leading to the cleavage of the 116-kDa polypeptide PARP into its characteristic 85-kDa fragment (Fig 1c). In our model, *hGH* gene transfer amplified this caspase-dependent pathway as a more marked increase in both activation of caspase-3, and cleavage of PARP was observed in U937-hGH cells when compared to control cells exposed to daunorubicin. All these results highlight an active process of cell death induced by *hGH*.

Decreased NF-κB activation in hGH-transfected cells in response to daunorubicin

We then decided to approach the role of NF-κB in this model. Indeed, daunorubicin is known to activate NF-κB,[19] and we have previously reported that hGH was able to alter this activation in LPS-stimulated human monocytes.[12] NF-κB DNA binding activity was tested in control and

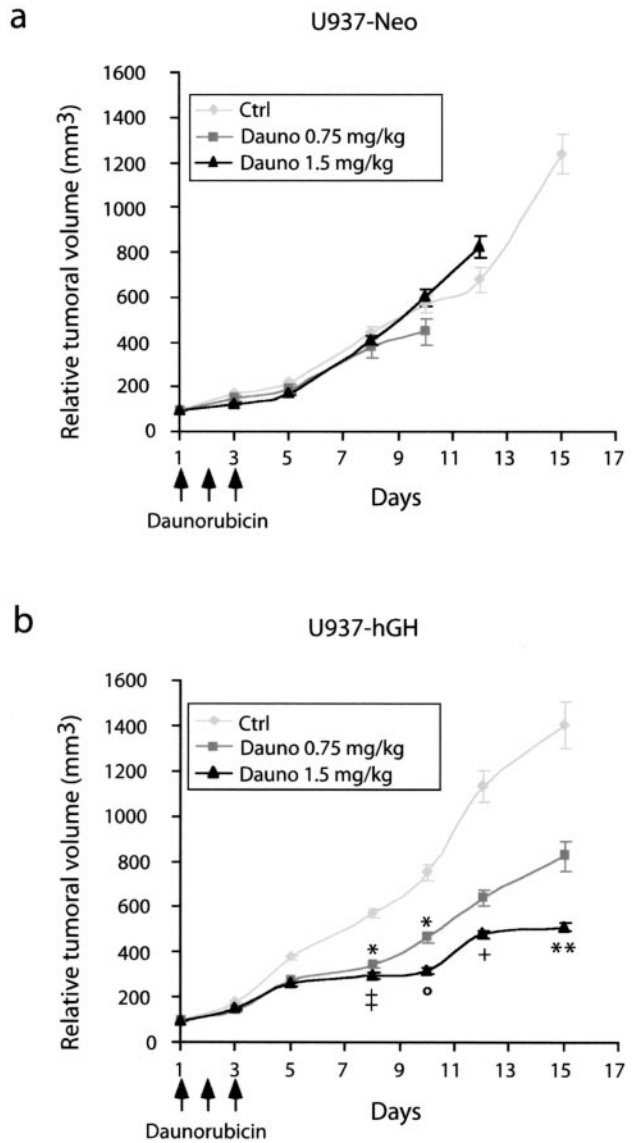

Figure 4 *In vivo* effect of *hGH* gene transfer on daunorubicin treatment of engrafted tumors. Irradiated nude mice were subcutaneously engrafted with U937-Neo (seven mice per group) or U937-hGH (10 mice per group) tumor fragments. Tumor-bearing mice were either left untreated or received i.p. injections of nontoxic doses of daunorubicin (0.75 and 1.5 mg/kg). These figures represent the relative tumoral volume (tumor volume at date *t*/ tumor volume at date 0) during the observation period, on the various treatments applied (*P=.05, **P=.03, +P=.01, ‡P=.002 and °P=.001, when comparing treated to untreated mice).

hGH-producing U937 cells exposed to daunorubicin (Fig 2a). The position of NF-κB was assessed by competition of nuclear extracts from daunorubicin-treated control U937 cells with nonradiolabeled consensus NF-κB (lane 5) or mutated NF-κB (lane 6) probes. As expected, a stronger protein–DNA complex was observed in nuclear extracts from control cells exposed to daunorubicin (lane 2) when compared to the untreated control cells (lane 1). In contrast, no variation in the signal intensity was observed when comparing untreated (lane 3) and daunorubicin-treated U937-hGH cells (lane 4). These results were confirmed by the densitometric analysis of the gel bands presented in Figure 2a.

To visualize increased retention of NF-κB in the cytoplasm of cells exposed to daunorubicin, permeabilized cells were stained with polyclonal Abs directed against the p65 subunit of NF-κB. As shown in Figure 2b, daunorubicin-treated U937-hGH cells exhibited few stained nuclei, whereas daunorubicin-treated control cells clearly exhibited intense nuclear staining (lower left panel). Untreated cells only showed a strong cytoplasmic staining.

Proteolytic degradation of IκB molecules precedes translocation of NF-κB to the nucleus.[20] To determine whether the decreased activation of NF-κB observed in treated U937-hGH cells was due to a modulation of IκB degradation, Western blot analyses were performed on cellular extracts from our cell lines. As depicted in Figure 2c, following daunorubicin exposure, degradation of IκBα was observed in U937-Neo cells, starting at 30 minutes of treatment, whereas no degradation was detected in U937-hGH cells for up to 2 hours of incubation.

Altogether these approaches therefore confirmed that hGH diminished transfer of NF-κB to the nuclei of daunorubicin-treated cells by inhibiting the degradation of IκB.

hGH gene transfer does not affect MDR of U937 cells

One of the major mechanisms involved in chemoresistance implies the activation of *multidrug resistance* (*mdr*) gene. It has been reported that NF-κB may be involved in the transcriptional regulation of *mdr-1* encoding P-gp,[21] the drug efflux pump, which normally participates in cell detoxification of various molecules such as anthracyclines.[22] In this line, we hypothesized that hGH would therefore allow entry of the drug into tumor cells by down-regulating P-gp expression, via the inhibition of NF-κB. This was tested by incubating our cell lines with a high dose of daunorubicin followed by simultaneous cytofluorometric analysis of intracellular accumulation of the drug (FL2 channel) and expression of membrane P-gp (FL1 channel), after staining with specific antibodies. We used MCF-7^{DXR} cells as the control, as they are resistant to doxorubicin, an analogue of daunorubicin. As illustrated in Figure 3, no variation of P-gp expression was evidenced in either U937-Neo or U937-hGH cells, when comparing the staining obtained with the anti–P-gp and the irrelevant Abs, and no decrease in drug accumulation was observed when comparing U937-hGH and U937-Neo cells ($\times$mean of 23.6 and 26.5 for U937-hGH and U937-Neo cells, respectively). In contrast, as expected, a lower drug accumulation was observed in MCF-7^{DXR} cells ($\times$mean of 11), together with intense P-gp expression. These results were also confirmed by RT-PCR analysis of mRNA extracted from these cells, with *mdr-1*-specific primers.[23] Under these conditions, no amplifications of the *mdr-1* mRNA were observed in either parental or hGH-transfected U937 cells, whereas an intense specific signal was detected in MCF-7^{DXR} cells (not shown).

In vivo *sensitization of tumors to daunorubicin by hGH gene transfer*

The therapeutic activity of *hGH* gene transfer was assessed *in vivo* in nude mice engrafted with either U937-Neo or U937-hGH tumors. Mice bearing 60–300 mm^3 subcutaneous tumors were assigned to three different groups: one group was not treated and the other groups received two subtoxic doses of daunorubicin. The drug was injected i.p. on three consecutive days. Drug toxicity was estimated during a preliminary experiment in non–tumor-bearing nude mice. As shown in Figure 4a, U937-Neo tumors remained insensitive to daunorubicin treatment regardless of the dose administrated. No further comparisons were possible after the 10th day due to the death of one animal in the group treated with 0.75 mg/kg daunorubicin. In contrast, Figure 4b shows an obvious dose-dependent response to this chemotherapy in U937-hGH tumors. The

Table 1 Variation in body weight during animal treatment

Daunorubicin (mg/kg)	U937-NEO			U937-hGH		
	Day 0	Day 10	Day 15	Day 0	Day 10	Day 15
0	25.57±0.17 (7)	26.80±0.17 (7)	28.24±0.29 (7)	26.77±0.16 (10)	27.43±0.18 (10)	28.09±0.18 (10)
0.75	25.34±0.25 (7)	26.17±0.21 (7)	27.77±0.27 (6)	26.65±0.17 (10)	27.06±0.15 (10)	26.96±0.15 (10)
1.5	25.54±0.34 (7)	25.40±0.41 (7)	22.92±0.28* (6)	25.92±0.17 (10)	24.98±0.19† (10)	23.24±0.26‡ (10)

Number in parenthesis represents the number of animals.
*P=.001 when comparing each value (in grams) in daunorubicin-treated groups to untreated groups.
†P<.01 when comparing each value (in grams) in daunorubicin-treated groups to untreated groups.
‡P<.001 when compared each value (in gram) in daunorubicin-treated groups to untreated groups.

growth rate of U937-hGH tumors was reduced by the two doses of daunorubicin (0.75 and 1.5 mg/kg). The observed effect was significant from the 8th day after initiation of treatment until the 15th day of observation, when comparing animals treated or not treated with daunorubicin. At day 10, the relative tumoral volume reached 753.6±36.2 mm^3 in untreated animals versus 469.8±23.1 mm^3 (n=10, P=.05) in animals treated with 0.75 mg/kg daunorubicin and 317.8±11.5 mm^3 (n=10, P=.001) in animals treated with 1.5 mg/kg daunorubicin. No further comparisons were possible after the 15th day due to the death of animals in the untreated group at day 18. The mean hGH concentration measured in sera of untreated and daunorubicin-treated animals was 0.57±0.51 ng/mL (n=26) and 9.82±7.48 ng/mL (n=27) for control and hGH-secreting tumor-bearing mice, respectively. Finally, no dramatic change in the body weight was observed for treated mice engrafted with either tumor (Table 1), indicating that the presence of the transgene did not induce higher toxicity.

Discussion

A large number of gene therapy protocols have been applied to cancer. Strategies have been developed to kill tumor cells directly by introducing suicide genes alone[24,25] or in combination with cytokines,[26] or genes encoding apoptotic proteins.[27,28] Alternatively, other authors have proposed strategies designed to boost the antitumor immune response. Tumor cells can be modified to express MHC class II and costimulatory molecules (for example B7-1).[29] As an alternative to tumor cells, normal dendritic cells treated *in vitro* with tumor-related peptide antigens[30] or transfected with cytokines[31] can also be used to elicit antitumor responses. However, the survey of ongoing gene therapy clinical trials using these methods did not show a clear benefit for patients with cancer.[32] In this condition, other genes should be considered, such as those enabling the improvement of drug effects in chemotherapy regimens. Up until now, only a few gene candidates have been successfully tested: the proapoptotic *bax* gene,[7] and, in a large panel of tumors, a gene encoding a negative dominant of IκB.[8–11] In the present study, we tried an alternative approach based on our previous results showing the capacity of *hGH* gene transfer to down-modulate NF-κB activation in response to bacterial lipopolysaccharides.[12] When using our *hGH*-transfected leukemia U937 cell lines, which produce human physiological concentration of hGH, ranging from 10 to 50 ng/mL,[14] we first found that these cells were rendered more sensitive to an apoptotic signal generated *in vitro* by the daunorubicin (Fig 1). This anthracycline, very frequently used in the treatment of various cancers, mediates its action by directly interacting with cell DNA. Higher sensitivity to daunorubicin was not correlated to an increased cell division in *hGH*-transfected cells, as we measured a similar growth rate in our two cell lines over a 3-day culture (not shown). This accentuation of cell death used the classical apoptosis-mediated pathway. Indeed, *hGH* gene transfer potentialized the activation of caspase-3 (CPP32) normally observed following anthracycline treatment,[33] and the resulting increased cleavage of PARP, one of the critical substrates that maintain cells alive.[34]

We then looked at NF-κB status in our cells, as anthracyclines are molecules inducing activation of NF-κB.[19] Gel shift assay or epifluorescence demonstrated that *hGH* affected NF-κB translocation into cell nuclei (Fig 2, a and b), and Western analysis supported the role of stabilization of IκB, the natural inhibitor of NF-κB[20] in this phenomenon (Fig 2c). In contrast to these results, NF-κB was equally activated in our control and in our different hGH-producing cell lines both exposed to 100 μM of ceramide (C2) (data not shown), excluding selection of U937 subtypes unable to respond to NF-κB inducers, as reported by others.[35] Additional experiments run with our cell lines incubated with paclitaxel (Taxol®), another anticancer drug inducing NF-κB activation,[20] clearly established a similar effect of *hGH* gene transfer in increased cell death (15.9±5.6% PI+U937-Neo cells to 32±3.5% PI+U937-hGH cells, mean value±SEM, P=.01 when comparing the two cell lines exposed to 5 nM paclitaxel, n=3; 32.8±2.7% PI+U937-Neo cells to 49.5±7.7% PI+U937-hGH cells, mean value±SEM, P=.02 when comparing the two cell lines exposed to 10 nM paclitaxel, n=3).

As NF-κB could control activation of the *mdr-1* gene,[21] which encodes the plasma membrane protein P-gp involved in the chemoresistance developed by a majority of tumors,[22] we postulated that *hGH* could mediate its accessory effect by down-regulating P-gp expression. However, this hypothesis was not confirmed, as flow cytometric determination of daunorubicin accumulation and P-gp expression did not reveal any difference between U937-Neo and U937-hGH cells (Fig 3). These data strongly support that chemoresistance may proceed via various mechanisms that could be controlled independently.

In view of our *in vitro* results, and to get some insights of the effect of *hGH* gene transfer on *in vivo* chemotherapy, as a model, nude mice were engrafted with our *ex vivo* engineered tumor cells. Then mice bearing tumors received i.p. injections of daunorubicin doses previously demonstrated to lack any toxicity in these animals. The growth of U937-hGH tumors started to decrease 8 days after the first injection of daunorubicin, compared to untreated U937-hGH tumors (Fig 4). Significant difference was observed after the 13th day. In contrast, similar treatment of mice bearing U937-Neo tumors demonstrated complete absence of cell response to the two doses of daunorubicin. It is of note that the apparent decrease in the relative tumoral volume of control tumor-bearing nude mice receiving 0.75 mg/kg daunorubicin at day 10 was no longer observed in the six surviving mice still followed over an 18-day period. Indeed, the slope of the growth curve of these surviving mice paralleled that of untreated control tumor-bearing nude mice (not shown). Moreover, monitoring of the body weight of the animals included in these experiments did not indicate any striking increased toxicity in any of the groups studied as assessed by the body weight survey (Table 1). Interestingly, serum concentration of circulating hGH was not affected by daunorubicin treatment because similar mean concentrations were found in the three groups of hGH-bearing tumor mice

at time of sacrifice, indicating a potent long-lasting effect of *hGH* gene transfer. From these *in vivo* experiments, we demonstrated that *hGH* gene transfer into tumor cells greatly improved their sensitivity to a cancer chemotherapy without any additional toxic effects.

Finally, although our approach is based on a treatment that must combine *hGH* gene transfer and an anticancer agent, its clinical value needs to be interpreted in the light of a study indicating a potential link between hGH therapy and mammary cancer in monkeys.[36] However, it must be remembered that, in this trial, monkeys were treated with high doses of exogenous hGH alone for 7 weeks, whereas we propose through gene transfer a moderate production of hGH. In addition, using transgenic mice overexpressing either bovine GH or rat prolactin, Wennbo and Tornell found mammary carcinoma in prolactin transgenic females only.[37]

In conclusion, although further experiments must be conducted to elucidate the mechanisms involved, *hGH*-based gene therapy may have significant potential for the treatment of chemoresistant tumors.

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