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Hepatocyte growth factor (HGF) protects c-met-expressing Burkitt's lymphoma cell lines from apoptotic death induced by DNA damaging agents

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Abstract

The relative sensitivity of neoplastic cells to DNA damaging agents is a key factor in cancer therapy. In this paper, we show that pretreatment of Burkitt's lymphoma cell lines expressing the *c-met* protooncogene with hepatocyte growth factor (HGF) protects them from death induced by DNA damaging agents commonly used in tumour therapy. This protection was observed in assays based on morphological assessment of apoptotic cells and DNA fragmentation assays. The protection was dose- and time-dependent — maximal protection requiring pre-incubation with 100 ng/ml HGF for 48 h. Western blotting analysis and flow cytometric studies revealed that HGF inhibited doxorubicin- and etoposide-induced decreases in the levels of the anti-apoptotic proteins Bcl-X_L, and to a lesser extent Bcl-2, without inducing changes in the pro-apoptotic Bax protein. Overall, these studies suggest that the accumulation of HGF within the microenvironment of neoplastic cells may contribute to the development of a chemoresistant phenotype.

Keywords: Hepatocyte growth factor; Burkitt's lymphoma cell lines; DNA damaging agents; Apoptosis

1. Introduction

The development of chemotherapy-resistant phenotypes is a major cause of treatment failure in numerous malignancies [1]. Undetectable subpopulations of tumour cells that survive drug-treatment consequently lead to relapse. It is commonly accepted that this subpopulation of drug resistant cells arises from genetic changes that occur in cancer cells [2]. However, the development of such phenotypes has also been attributed to a number of other causes, including the inability to metabolise cytotoxic drugs, mutations in the *TP53* tumour suppressor gene [3] and the apoptosis suppressing effects of certain growth factors and cytokines [4,5]. As apoptosis is a major mechanism by which DNA-damaging agents kill tumour cells [6], it is readily appreciated that abnormal accumulation of certain cytokines, hormones and growth factors within tumours may contribute to the development of drug-resistant phenotypes.

Hepatocyte growth factor (HGF) is a multifunctional factor of mesenchymal origin that acts as mitogen, motogen and morphogen depending on the cell target and cellular context [7,8]. HGF also causes the spread of epithelial cells, a property that led to the original designation 'scatter factor' [9], a term which is still used synonymously with HGF. The receptor for HGF is the product of the *c-met* proto-oncogene which has characteristics of a tyrosine kinase receptor [10]. Evidence suggests that the HGF/*c-met* system not only plays an essential role in liver development and regeneration, but also is a crucial modulator in a number of other organs. Apart from the above functions in normal physiological processes, there is also ample evidence for a key role of the HGF/*c-met* pathway in tumour growth, invasion and metastasis [11]. Several recent studies have also suggested that HGF may modulate the process of apoptosis. Thus, HGF prevents cell death induced by inhibition of adhesion [12,13], promotes survival and differentiation of hepatocytes during development and acts as a survival factor for the rat pheocromocytoma cell line (PC12) [14]. Evidence has also emerged that it protects epithelial and carcinoma cells against apoptosis induced by cytotoxic drugs, gamma radiation and ultraviolet (UV) light [15].

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Recently, it has been shown that stromal cells in lymphoid organs such as the bone marrow, tonsils and lymph nodes produce HGF [16,17]. Furthermore, the c-met receptor is expressed by B lymphocytes in germinal centres, as well as on lymphoma and myeloma cells from patients and cell lines [16,18,19].

Most of the information on the role of HGF in apoptotic processes has been obtained on epithelial cells [15,20], and therefore does not necessarily apply to B cells. Taking this into account, we decided to test whether, as in epithelial and carcinoma cells, HGF protects Burkitt's lymphoma cell lines expressing functional c-met receptor from apoptosis. We show that pretreatment of c-Met-positive cell lines (Raji and EB4) with HGF protects them against apoptotic cell death induced by various chemotherapeutic drugs used in the therapy of malignant diseases. In contrast, similar pre-treatment had no effect on apoptosis induction in a c-met negative cell line (Namalwa). Together, this survival promoting mechanism may prevent drug-induced cell death resulting in pleiotropic drug resistance.

2. Materials and methods

2.1. Antibodies and reagents

Antibodies used in this study were: rabbit anti-human c-met, rabbit anti-Bcl-2, rabbit anti-Bcl-X_L, rabbit anti-phosphotyrosine, goat anti-rabbit IgG conjugated with horseradish peroxidase (Santa Cruz Biotechnology, Santa Cruz, CA, USA); mouse monoclonal antibodies: anti-c met (DO24) (Upstate Biotechnology, Lake Placid, NY, USA), anti-Bcl-2 (Sigma, Poole, UK), anti-Bcl-X_L (Chemicon, Harrow, UK) anti-Bax (Santa Cruz Biotechnology). Doxorubicin, etoposide and actinomycin D were obtained from Sigma (Poole, UK). They were dissolved in 70% ethanol and kept as stock solutions at −20°C. Human recombinant HGF was purchased from R&D Systems (Abingdon, Oxon, UK). Roswell Park Memorial Institute (RPMI) 1640 medium and all other components of culture media were purchased from Sigma.

2.2. Cell lines and culture conditions

The Raji cell line was obtained from the European Collection of Animal Cell Cultures (ECACC, Porton Down, UK). The Namalwa cell line was a kind gift from J. Ross, Department of Clinical and Surgical Sciences, University of Edinburgh, UK. R. Jefferis, University of Birmingham, UK kindly provided the EB4 cell line. A431 epidermoid carcinoma cell line which constitutively expresses c-met came from ECACC. The cells were grown in RPMI 1640 medium supplemented with 10% (v/v) fetal bovine serum, 2 mM L-glutamine, 100 μg/ml streptomycin, 100 units/ml penicillin, at 37°C in 5% CO_2/95% air.

2.3. Treatment of cells with DNA damaging agents

For most experiments, subconfluent proliferating cultures were preincubated in the presence or absence of HGF for 48 h and then sham-treated or treated with a DNA damaging substance (concentrations indicated in figures). After exposure to DNA damaging agents (2 h), the cells were washed twice and then incubated in fresh medium again with or without HGF at 37°C for 24 h. Cells were finally harvested for apoptosis assays. In some experiments, instead of HGF, monoclonal agonistic anti-c-met antibody was used (DO-24 from Upstate Biotechnology).

2.4. Morphological assessment of apoptosis

Morphological evaluation was performed by mixing 10 μl of a 1×10^6 cells/ml suspension with 10 μl of ethidium bromide (100 μg/ml) and acridine orange (100 μg/ml) dye solution and examining the cells by fluorescence microscopy in a blind fashion. At least 200 cells were scored for each treatment. The percentage of apoptotic cells was determined from the sum of cells showing bright green or bright orange chromatin, which was highly condensed or fragmented.

2.5. DNA fragmentation assays

Cells were washed twice in phosphate-buffered solution (PBS) and lysed with 100 μl of lysis buffer (10 mM Tris–HCl pH 7.4, 10 mM ethylene diamine tetra acetic acid (EDTA), 0.5% Triton X-100). After 10 min at 4°C, the lysates were centrifuged at 12000*g* for 30 min at 4°C. The supernatants containing fragmented chromatin were incubated with ribonuclease A (0.4 mg/ml) for 1 h at 37°C and then with proteinase K (0.4 mg/ml) for 1 h at 37°C. DNA fragments were then precipitated by adding NaCl and isopropanol at final concentrations of 0.5 M and 50% (v/v), respectively. After centrifugation, the pelleted DNA fragments were dissolved in water and subjected to electrophoresis in 1.5% agarose in Tris–HCl-EDTA buffer, pH 8.0. DNA in the gels was visualised under UV light after staining with 0.5% ethidium bromide.

2.6. Flow cytometric analysis

The expression of Bcl-2 and Bcl-XL was monitored by staining of saponin-permeabilised cells with monoclonal antibodies anti-Bcl-2 or Bcl-X_L (both at 5 μg/ml) for 30 min at 4°C. Cells were also stained with isotype-matched normal mouse IgG control. Goat anti-mouse IgG antibodies conjugated with phycoerythrin were

used as the second antibody (Sigma). Cells were analysed on an EPICS XL flow cytometer (Coulter, Miami, FL, USA).

2.7. Western blotting analysis

Cells were harvested by centrifugation, washed twice with cold PBS and lysed at 4°C with 100 μl/10^6 cells of lysis buffer (PBS containing 1% Nonidet P40 (NP40), 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulphate (SDS) (Sigma) and a cocktail of protease inhibitors (Merck, Lutterworth, UK). The protein content of the lysates was determined using a Bio-Rad microassay (Bio-Rad, York, UK). Total cell lysates (20 μg/lane) were electrophoresed on 10% SDS-polyacrylamide gels after boiling samples for 5 min in Laemmli sample buffer. Proteins were transferred electrophoretically onto Immobilon membranes (Millipore, Bedford, MA, USA). After electrotransfer, the membranes were blocked with PBS-Tween buffer containing 5% (w/v) non-fat dry milk, washed with PBS Tween buffer and incubated with the primary antibody diluted in blocking buffer overnight at 4°C. Membranes were then washed, incubated with peroxidase-conjugated detector antibody for 2 h (goat anti-rabbit IgG, Santa Cruz Biotechnology) and re-washed. Blotted proteins were detected using the enhanced chemiluminescence (ECL) detection system (Amersham Pharmacia Biotech, Little Chalfont, Bucks, UK). Each western blotting experiment was repeated four times.

2.8. c-met Phosphorylation assays

For c-met phosphorylation experiments, cells were incubated overnight in serum-free medium in the presence or absence of 150 ng/ml human recombinant HGF (R&D Systems). After that, the cells were solubilised in cold 20 mM Tris–HCl buffer, pH 8.0 containing 250 mM NaCl, 20% glycerol, 2% NP40, 20 μg/ml aprotinin, 20 μg/ml leupeptin, 10 mM EDTA, 4 mM sodium orthovanadate and 10 mM sodium fluoride. After 30 min at 4°C, the lysates were centrifuged at 10 000*g* for 30 min after which the supernatant was precleared with protein A sepharose CL-4B (Pharmacia Biotech) for 1 h at 4°C. After centrifugation, the supernatant was incubated with rabbit anti-c-met antibody coupled to protein A-Sepharose for 3 h at 4°C. The beads were washed two times with lysis buffer and then boiled in Laemmli sample buffer for 5 min. The eluted proteins were resolved by 8% SDS-polyacrylamide. Western blotting was performed as described above.

2.9. Statistical evaluation

The data are presented either as means ± standard deviation (S.D.) or as means ± standard error of the mean (SEM) of n experiments. Student's paired t-test was used to compare the response between two treatments. All experiments described were performed on a minimum of three occasions with cultures being set up in triplicate in every experiment.

3. Results

3.1. Some Burkitt's lymphoma cell lines express functional c-met receptor

Burkitt's lymphoma cell lines were tested by western blot analysis for the presence of c-met protein in the cell lysates. The A431 epidermoid carcinoma cell line known to express high levels of the c-met receptor was used as positive control. Of the cell lines tested, EB4 and Raji expressed high levels of c-met, while the Namalwa cell line was on all occasions negative. In the next stage of experiment, we attempted to test whether the receptor expressed by the cells was functional, i.e. whether it gets phosphorylated on tyrosine residues in response to HGF stimulation. To this end, cell lines were incubated with HGF and then immunoprecipitated c-met was analysed for phosphorylation. The experiments demonstrated that HGF is able to induce c-met phosphorylation in c-met-positive Burkitt's lymphoma cell lines indicating that the HGF/c-met pathway is capable of signalling (Fig. 1). These results confirm findings reported previously [16,19].

3.2. HGF protects Raji and EB4 Burkitt's lymphoma cells from apoptosis induced by several DNA damaging agents

Most data on c-met signalling have been obtained on epithelial cells and hence do not necessarily apply to B cells. Therefore, in order to test whether HGF also protects Burkitt's lymphoma from apoptotic cell death, we used two cell lines which constitutively express the *c-met* proto-oncogene-encoded HGF receptor — namely Raji and EB4. These cell lines did not produce HGF as analysed by a sandwich immunoassay of conditioned cell culture supernatants (data not shown). In parallel, experiments were conducted with the Namalwa cell line which does not express the HGF receptor. The effects of HGF on apoptosis induced by exposure to a number of DNA damaging agents was examined. We found that pretreatment with 100 ng/ml of HGF for 48 h effectively protected EB4 and Raji cells from apoptotic cell death induced by etoposide, doxorubicin and actinomycin D. In contrast, the HGF receptor-negative Namalwa cell line was not protected from apoptotic cell death by HGF. These results were confirmed using DNA fragmentation assays (Fig. 2). Subsequent experiments revealed that protection was dependent on both the

dose of HGF employed and pre-treatment time. Thus, using a 48 h pre-incubation time, a dose of 10 ng/ml exerted a small, but statistically significant protection, while the maximal effect was obtained at 300 ng/ml. A dose of 100 ng/ml was used in subsequent pre-incubation time course studies. These experiments revealed little or no protection when HGF was added to cultures at the time of doxorubicin treatment (data not shown). The degree of cytoprotection increased with pre-incubation times reaching a maximum when cells were incubated with HGF for 48 h before doxorubicin treatment (Fig. 3). Therefore, a 48 h pre-treatment with 100 μg/ml HGF was used in subsequent experiments.

3.3. HGF protection requires c-met receptor expression

To further ascertain if the HGF-mediated protection of target cells requires the expression of the c-met receptor, experiments similar to those described above were performed with an anti-c-met monoclonal agonistic antibody, DO-24. This antibody recognises an extracellular epitope on the c-met receptor and triggers all the biological effects elicited by HGF, namely motility, proliferation, cell survival, invasion, tubologenesis and angiogenesis [21]. Pre-incubation with the antibody alone prior to doxorubicin treatment protected EB4 cells against apoptosis, 1 μg of the antibody giving cytoprotection equivalent to 100 ng of human recombinant HGF. No protection was observed when the cells were cultured in the presence of an irrelevant control antibody of the same isotype (Fig. 4). Similar results were obtained using Raji cells. In contrast all these treatments failed to protect the c-met-negative Namalwa cell line (data not shown). Collectively, with experiments showing HGF-induced c-met phosphorylation, these data show that HGF-mediated protection against drug-induced apoptosis is dependent upon the expression of the c-met receptor. Most DNA damaging agents target cycling cells and one possible explanation for the effects of HGF on drug-induced effects in Burkitt's lymphoma cell lines would be the regulation of proliferation. However, HGF tested over a wide range of concentrations showed no effect on [^{3}H]-thymidine incorporation in the cell lines under study (data not shown).

3.4. The rescue from apoptosis produced by pre-incubation with HGF is associated with alterations in the levels of apoptosis regulatory proteins

The molecular pathways that regulate apoptosis are not completely elucidated, although several proteins seem to be involved in the control of this process. Included amongst these are the Bcl-2 family of proteins which serve as critical regulators of pathways involved in apoptosis, acting to either inhibit or to promote cell death. Cell death decisions may depend on the relative abundance of anti-apoptotic (e.g. Bcl-2, Bcl-X_L) and pro-apoptotic proteins (e.g. Bax). Altered expression of these proteins occurs commonly in human cancers, contributing to neoplastic cell expansion by suppressing programmed cell death and extending the tumour life span [22]. Moreover, B cells that survive and emerge from germinal centres express Bcl-2 [23]. The importance of the Bcl-2 family in apoptosis control prompted us to study the effect of HGF on the expression of Bcl-2 and Bcl-X_L (anti-apoptotic) and Bax (pro-apoptotic) proteins following the addition of DNA damaging agents inducing apoptosis. To this end, cells were pre-incubated without or with HGF (100 ng/ml) and subsequently treated with DNA damaging agent following which expression of the Bcl-2, Bcl-X_L and Bax proteins was determined by western blot analysis and flow cytometry. Western blotting revealed a decreased Bcl-2 expression in doxorubicin treated cells in comparison to untreated control cells. However, pretreatment with HGF consistently prevented the increase of Bcl-2 expression,

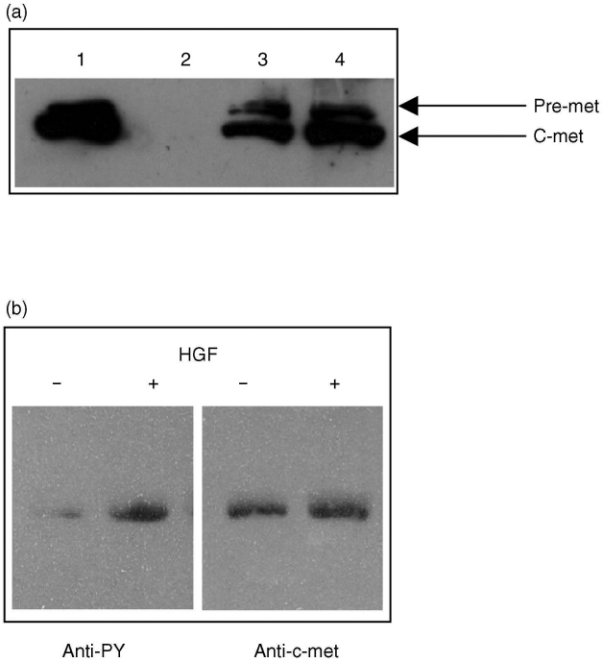

Fig. 1. Burkitt's lymphoma cell lines express a functional c-met receptor. (a) Burkitt's cell lines (EB4, Raji and Namalwa) were harvested at the logarithmic phase of growth and lysed with lysis buffer containing protease inhibitors. Resulting lysates were resolved by SDS-polyacrylamide electrophoresis. Gels were electroblotted onto Immobilon membranes then stained with rabbit anti-c-met antibody followed by goat anti-rabbit antibodies conjugated with horseradish peroxidase. The reaction was visualised by the enhanced chemiluminescence (ECL) system. 1, A431 epidermoid carcinoma cell line (positive control); 2, Namalwa; 3, Raji; 4, EB4 cells. The c-met precursor (pre-c-met) and c-met β chain are indicated. (b) Tyrosine phosphorylation in EB4 cells in response to cultivation with hepatocyte growth factor (HGF). C-met was precipitated with anti-c-met antibodies and the western blot was consecutively stained with anti-phosphotyrosine (anti-PY) or anti-c-met antibodies.

although the effect was small. The expression of Bcl-X_L protein, another member of the Bcl-2 protein family with anti-apoptotic properties, was also markedly reduced following doxorubicin treatment. Once again pretreatment with HGF ablated the doxorubicin-induced decrease in the levels of Bcl-X_L. However, no effect of HGF on the expression of the pro-apoptotic Bax protein was observed. These results were confirmed by immunofluorescent staining of the cells for Bcl-2 and Bcl-X_L and subsequent flow cytometric analysis (Fig. 5). Similar results were obtained using etoposide (40 μM) as DNA damaging agent (data not shown).

4. Discussion

HGF is widely recognised for its role as a mitogenic, morphogenic and motogenic factor. Its role in apoptosis has also been recognised, but less widely studied. Interestingly most of the studies performed to date demonstrate the ability of HGF to initiate apoptosis in a variety of cell types, presumably through activation of protein kinase C [24]. In contrast, a few studies also reveal that HGF can act as a negative regulator of programmed cell death, however the underlying mechanisms remain poorly defined [15,20].

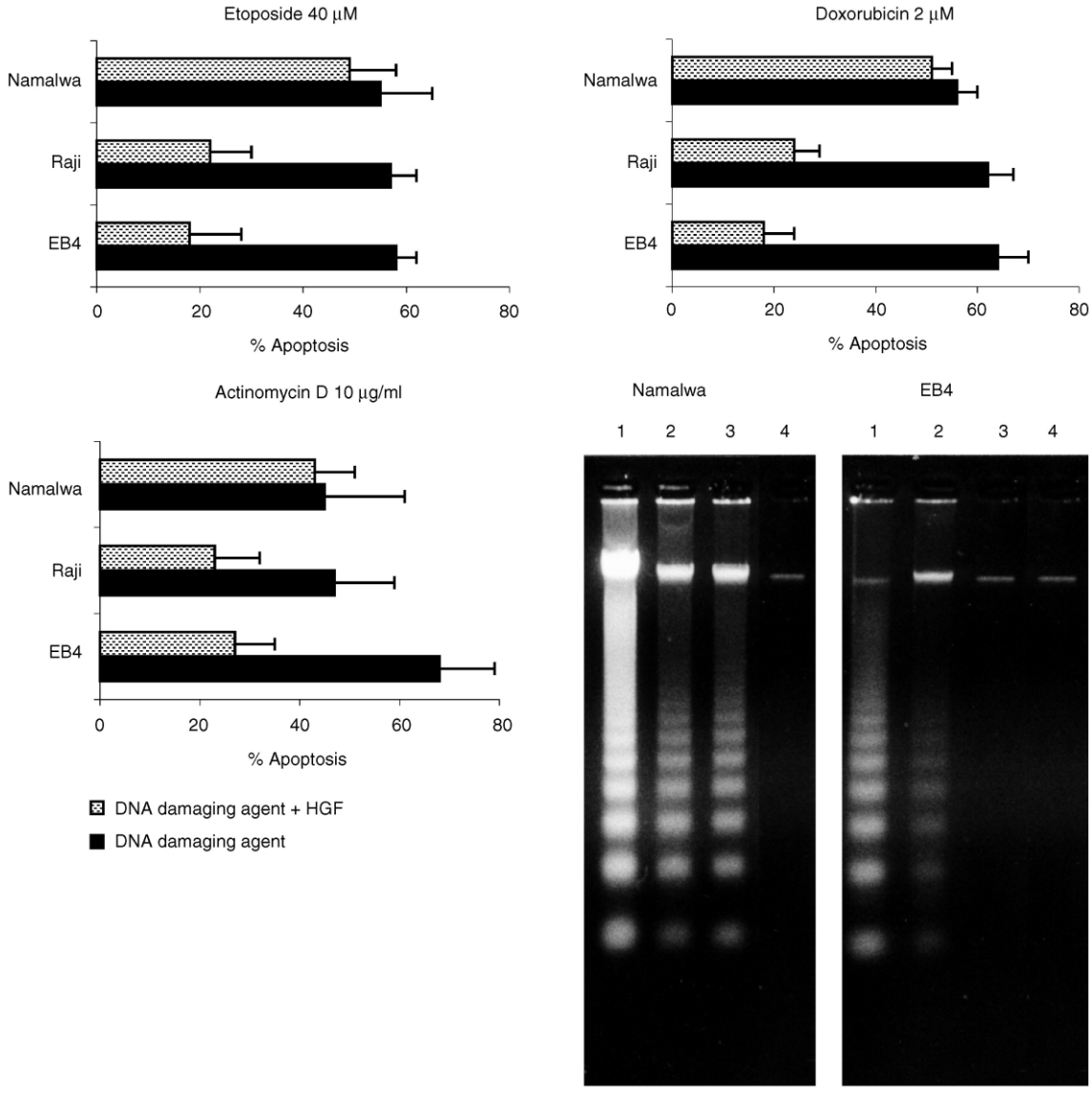

Fig. 2. Hepatocyte growth factor (HGF) protects c-met-expressing Burkitt's lymphoma cell lines against apoptosis induced by a variety of DNA damaging agents. (a) Cells were pre-incubated for 48 h with (HGF) (100 ng/ml) and then treated with DNA damaging agent used at optimal doses established in background studies. Apoptosis was scored in a blind fashion by fluorescent microscope examination of acridine orange/ethidium bromide-treated cells. Results of five independent experiments are presented and expressed as means ± standard deviation (S.D.). (b) Cells treated in the same way as in (a) were lysed, DNA extracted and DNA fragmentation assessed by electrophoresis. Lane 1, cells incubated with doxorubicin (2.0 μM); lane 2, cells incubated with HGF (10 ng/ml) and then with doxorubicin, lane 3, cells incubated with HGF (100 ng/ml) and then with doxorubicin; lane 4, cells incubated with HGF (100 ng/ml).

Based on these observations, we decided to investigate whether HGF treatment could protect Burkitt's lymphoma cell lines from apoptosis. The lines selected undergo vigorous apoptotic death after appropriate stimulation as assessed by morphological examination, and DNA laddering analyses. Furthermore, two of lines, namely Raji and EB4, constitutively express functional c-met receptor, while Namalwa does not. As such these cell lines provided an ideal model to investigate the putative modulatory actions of HGF on drug-induced apoptosis.

The present study strongly suggests that HGF is indeed able to drastically reduce the level of apoptosis initiated in these cell lines as a result of DNA damage. The results also indicate that the anti-apoptotic protection observed required signal transduction from the c-met receptor since little or no cytoprotection was observed in the case of the Namalwa cell line that does not express the c-met receptor. Further support for the role of the c-met receptor was provided by studies revealing that an agonistic monoclonal antibody recognising the extracellular portion of c-met was capable of mimicking the cytoprotective action of HGF. Together with results showing c-met phosphorylation in response to HGF, the data presented in this work provide at least strong circumstantial evidence for a HGF/c-met interaction as the basis for the observed effects. Maximal HGF-mediated protection required a prolonged exposure of 48 h, suggesting that the protective mechanism requires new protein synthesis. It should be noted that all the different assays of cell survival and cytotoxicity used revealed HGF-mediated cytoprotection, although the magnitude of cytoprotection differed from assay to assay. This most probably reflects the fact that the assays employed measured different end-points.

The mechanisms of HGF-induced protection of c-met-expressing cells is complex and may involve inhibition of several different pathways. Our studies suggest that one of the ways HGF may exert its protective effect is by targeting the anti-apoptotic mitochondrial pore forming protein Bcl-X_L, and to a lesser extent Bcl-2 protein. In this connection, it should be noted that DNA damaging agents are known to induce apoptosis through this pathway [25]. The Bcl-2 protein, originally discovered by Tsujimoto and Croce [26] is an intracellular integral membrane protein that resides in the outer mitochondrial membrane, nuclear envelope and parts of the endoplasmic reticulum. The Bcl-2 family members such as Bcl-X_L and Bcl-2 are known to protect cells against apoptosis induced by a variety of stimuli [27,28]. Our studies identify one of many possible apoptosis-induction pathways that may be modulated by HGF. Most significantly HGF abrogated the marked doxorubicin-induced decrease in the expression of Bcl-X_L. Bcl-2 expression was also protected however to a lesser

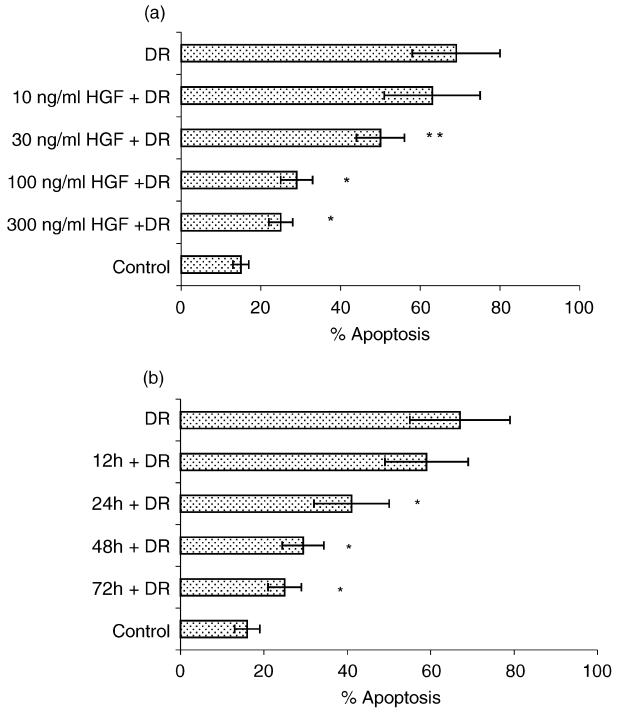

Fig. 3. Hepatocyte growth factor (HGF) protection against apoptosis induced by the DNA damaging agents is dose- and time-dependent. (a) Cells were pre-treated with various concentrations of HGF for 48 h and then treated with doxorubicin (2.0 μM) (DR); $^{*}P<0.02$, $^{**}P<0.05$. (b) Cells were pretreated with 100 ng/ml HGF for different time periods before treatment with doxorubicin. In both cases, apoptosis was assessed by fluorescence microscope examination of cells stained with ethidium bromide/acridine orange. Results are expressed as the mean ± standard error of the mean (S.E.M.) of 5 experiments, $^{*}P< 0.02$, $^{**}P<0.05$.

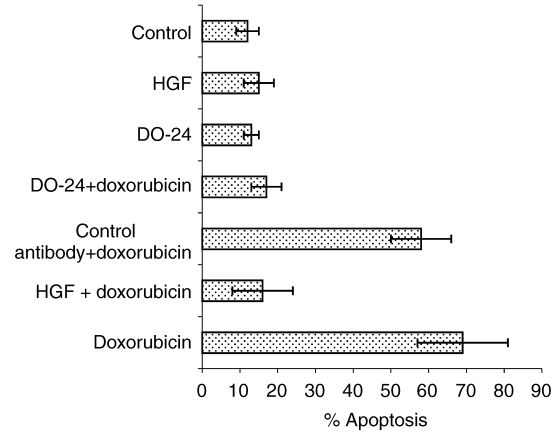

Fig. 4. Mimicking of hepatocyte growth factor (HGF)-mediated protection of EB4 cells against doxorubicin induced apoptosis by a specific antibody recognising the extracellular portion of c-met. Subconfluent cultures of EB4 cells were pretreated with HGF and/or agonistic monoclonal antibody (DO-24, 1 μg/ml) for 48 h, than exposed to doxorubicin (2.0 μM for 1 h) and then incubated for a further 24 h in doxorubicin-free medium. As a control, an isotype-matched mouse monoclonal antibody of the same concentration was used. Cells were then harvested, stained with ethidium bromide/acridine orange dye and examined for apoptotic cells under the fluorescent microscope. Monoclonal agonistic antibody binds to an extracellular portion of the c-met receptor and triggers all the biological effects elicited by HGF [21].

degree. Whether or not this is due to an inhibition of Bcl-2/Bcl-X_L degradation remains to be established. Nevertheless, the resultant effect may be to render cells more resistant to a variety of DNA-inducing agents, by blocking the induction of apoptosis downstream of DNA damage. These studies also strongly suggest that the accumulation of HGF within neoplastic cell tissue environments may contribute to the development of a chemoresistant phenotype. In this regard, it should be noted that stromal cells from bone marrow and secondary lymphoid tissues can secrete substantial amounts of HGF [16,17,29] and this influences proliferation, differentiation and adhesion of normal B cells. Furthermore, enhanced levels of HGF have been reported in the blood, bone marrow, plasma and pleural fluid of patients suffering from lymphomas [30,31] and have been shown to promote the adhesion of lymphoma cells to extracellular matrix proteins [19]. Concentrations of HGF of the same order were shown to protect cells from apoptosis in this work.

Observations in several other types of cells support the hypothesis of HGF as an important modulator of apoptosis. Wong and coworkers recently reported that HGF promotes motor neurone survival and this effect of HGF is synergised with ciliary neurotrophic factor [32]. HGF has also been shown to prevent liver progenitor cell apoptotic death *in vitro* [20] and targeted expression of c-met in the liver of transgenic mice renders their hepatocytes constitutively resistant to apoptosis and permits immortalisation of these cells [33]. Finally, HGF protected renal epithelial cells from apoptosis [34]. In this paper, we extend these observations and show that HGF can also modulate apoptosis within the lymphoid system.

Nevertheless, while we feel that overall our observations provide evidence that as in other systems, in the conditions employed in this work HGF protects lymphoma cells from apoptosis induced by chemotherapeutic agents. The possibility that it also inhibits non-apoptotic-induced cell death cannot be excluded.

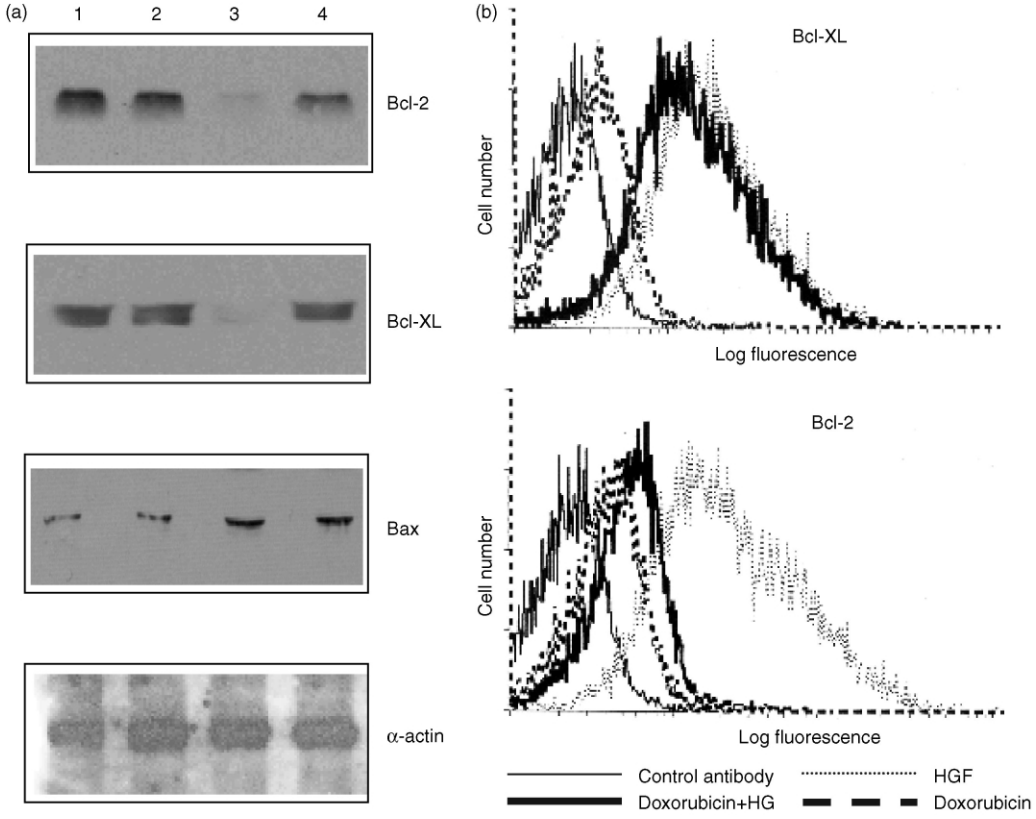

Fig. 5. Western blot and flow cytometric analysis of Bcl-2 and Bcl-X_L expression in EB4 cells treated with doxorubicin and doxorubicin together with hepatocyte growth factor (HGF). (a) Cells were pretreated with HGF (48 h) and then with doxorubicin (2 h) as described in Materials and methods. After exposure to DNA damaging agent, cells were washed twice and then incubated in fresh medium with or without HGF for 24 h. After that, a proportion of cells were lysed and equal aliquots of protein were analysed on 8% SDS-polyacrylamide gels, transferred to Immobilon membranes and immunoblotted with the appropriate antibodies. On all occasions, equal protein loading was confirmed by blotting for α-actin. Lane 1, control (cells incubated in medium only); lane 2, cells preincubated with HGF; lane 3, cells preincubated in medium without HGF then treated with doxorubicin; lane 4, cells pretreated with HGF and then treated with doxorubicin. (b) The cells, treated as described above were washed, permeabilised and stained with anti Bcl-2 or anti-Bcl-X_L specific monoclonal antibody followed by phycoerythrin-conjugated goat anti-mouse. Isotype-matched monoclonal antibodies were used as controls. The cells were analysed by flow cytometry. Both flow cytometric and immunoblotting were performed three times. Results of a representative experiment are presented.

Obviously measurements of the effect of HGF on other markers of apoptosis such as caspase activation or caspase-targeted cleavage will be necessary to resolve this.

In conclusion, we have shown that HGF promotes the survival of c-met-expressing Burkitt's lymphoma cell lines *in vitro*. A similar interaction with stromal cell-derived HGF *in vivo*, could provide neoplastic cells expressing the HGF receptor with a survival advantage and thus influence the disease outcome in patients on chemotherapy. As recently stressed by the elegant experiments of Taylor and colleagues [35], the role of such a microenvironmentally-produced factor in the emergence of chemoresistant phenotype must be understood, if we are to develop more effective strategies for therapeutic intervention.

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