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A tumour stage-dependent evolution of drug resistant T cell lymphoma: Role of soluble mediators of tumour and host origin

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ABSTRACT

The present investigation was carried out to investigate if soluble mediators present in tumour microenvironment and systemic circulation of a tumour-bearing host can regulate growth properties and response of the cells of a T cell lymphoma to chemotherapeutic drug: cisplatin, depending on the stage of tumour progression. In order to investigate this, tumour cells of a murine T cell lymphoma, designated as Dalton's lymphoma (DL), were incubated *in vitro* for 48 h in the presence of ascitic fluid and serum obtained from cisplatin treated or untreated tumour hosts at early or late tumour-bearing stages and cell survival was estimated. It was observed that tumour serum and ascitic fluid showed a tumour stage-dependent differential ability to regulate tumour cell survival and susceptibility of the tumour cells to the cytotoxic action of cisplatin. A tumour stage-dependent qualitative and quantitative difference in the profile of cell survival regulating cytokines: IL-1, IL-2, IFN-γ, TNF-α, VEGF and TGF-β in the ascitic fluid and serum of the tumour-bearing host was observed to be associated with a tumour stage-dependent differential regulation of survival of tumour cells by modulation in the expression of growth regulating proteins: IL-2R, p53, CAD, Hsp70 and Bcl-2. Further the result also showed that production of IL-1, TNF-α, and NO by macrophages could be implicated in the differential action of tumour sera on the altered survival responses of tumour cells depending on the stage of tumour growth. Possible mechanisms involved in the tumour stage-dependent differential survival response of tumour cells and evolution of drug resistance are discussed. The finding of this investigation will have clinical implications in designing of therapeutic strategies for T cell lymphoma based on manipulation of tumour growth regulatory mediators present in the tumour microenvironment.

1. Introduction

Outcome of a successful treatment strategy for cancer is dependent on a multitude of host and tumour-derived factors [1–2], which need to be understood precisely in a tumour-specific and tumour stage-dependent manner. Moreover, the participation of such mediators in the modulation of the response of tumour cells to the cytotoxic action of chemotherapeutic drugs remains unclear in case of most of the human malignancies. Recently, we reported that cells of a T cell lymphoma show a differential susceptibility to the cytotoxic action of chemotherapeutic drugs: cisplatin and doxorubicin depending on the stage of tumour progression [3]. However, owing to the tremendous complexity of mediators involved in host–tumour interactions, a definition of such factors in the tumour microenvironment and in the systemic circulation of the tumour-bearing host has remained elusive. Reports from our and other laboratories have indicated the existence of a numerous serum-borne factors that are implicated in the regulation of cell survival of a variety of cell types [4–7]. Nevertheless, such systemic mediators are also reported to be involved in the differential regulation of bone marrow cell survival and hematopoiesis [8–10]. Thus it is likely that such serum-borne soluble mediators may also have an indispensable role to play in the regulation of tumour growth and susceptibility of tumour cells to chemotherapeutic drugs.

Murine tumours of spontaneous origin have been reported to resemble with human malignancies most closely. Therefore, they serve as ideal tumour models for various investigative purposes to understand host–tumour relationship and for the designing and testing of therapeutic strategies [11]. In order to understand the dimensions of host–tumour interactions in a host bearing a progressively growing T cell lymphoma, we have been working on a

Abbreviations: IL-1, interleukin; IL-2, interleukin 2; IL-10, interleukin 10; IL-2R, interleukin 2 receptor; VEGF, vascular endothelial growth factor; TGF-βtumour, growth factor beta; IFN-γ, interferon gamma; CAD, Caspase-activated DNase; DL, Dalton's lymphoma; Hsp70, Heat shock protein 70; DLAF, DL ascitic fluid; CP, cisplatin.

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murine model of a transplantable T cell lymphoma of spontaneous origin designated as Dalton's Lymphoma (DL) [3,12,13]. T cell lymphomas constitute around 6% of the total malignancies and are considered as one of the major malignancies of hematological origin with complex host–tumour relationship, difficult for clinical management [14]. The DL can grow both in the form of an ascitic and a solid tumour and has been reported to possess chromosomal aberrations [15]. Like some other lymphomas and leukemias of human origin, DL cells do not metastasize to other lymphoid organs [16]. During the course of our previous investigations, we have observed that the progression of ascitic DL growth is rapid in syngenic BALB/c ($H2^d$) mice, causing death of the host in a relatively short time [3]. Further, DL growth has been shown to be associated with the onset of thymic atrophy, modulation of macrophage antitumour activity and other cellular and humoral immune responses associated with an alteration of Th1/Th2 cytokine balance [17–19]. Recently, we have also demonstrated that interactions between the immune, endocrine and nervous systems play an important role in determining host–tumour relationship in mice-bearing DL [12,13,15,19]. However, serum-borne soluble mediators capable of differentially influencing the cytotoxicity of chemotherapeutics drugs, remains to be worked out.

In view of the above-mentioned observations, in the present study, we were interested to define such soluble mediators of local tumour microenvironment *vis a vis* other systemic factors capable of differentially regulating the response of DL to the cytotoxic action of chemotherapeutic drugs.

2. Materials and methods

2.1. Mice and tumour system

Pathogen-free inbred adult mice of BALB/c ($H\text{-}2^d$) strain were used at 8–12 weeks of age. The mice received food and water *ad libitum* and were treated with utmost human care in an approved and certified animal room facility of the Banaras Hindu University at the Institute of Medical Sciences. Dalton's lymphoma (DL) is being maintained in ascitic form by serial transplantation in BALB/c mice or in an *in vitro* cell culture system by serial passage. Irrespective of whether the DL cells were obtained from the *in vitro* culture system or from the ascitic fluid they exhibited similar phenotypic features. A stock of DL cells is also maintained in a cryopreserved state for reference. Mice were transplanted i.p. with 1×10^5 cells/mouse, in 0.5 ml phosphate buffered saline (PBS) which normally survives for 20 ± 2 days.

2.2. Reagents

Tissue culture medium RPMI 1640 was purchased from Hyclone (Logan, Utah, USA). The culture medium were supplemented with 20 μg ml^{-1} gentamycin, 100 μg ml^{-1} streptomycin, 100 IU penicillin purchased from Himedia (Mumbai, India) and 10% fetal calf serum from Hyclone (Logan, Utah, USA). Antibodies against IL-1, IL-2, IL-2R, IFN-γ, TNF-α, TGF-β, Hsp70, VEGF, p53, Bcl-2, β-actin and Caspase-activated DNase (CAD) were purchased from Imgenex (San Diego, CA, USA) and Chemicon (Hampshire, UK). Secondary antibodies conjugated to alkaline phosphatase were obtained from Bangalore Genie (Bangalore, India). BCIP/NBT was purchased from Amresco (Solon, Ohio, USA). All the reagents were free from endotoxin contamination.

2.3. Protocol for in vivo treatment

Mice in-group of six each was transplanted i.p. with DL (1×10^5 cells/mouse in 0.5 ml of PBS). DL ascitic fluid (DLAF) or serum was harvested from mice on day 5 and 17 after the transplantation; henceforth referred to as early and late tumour-bearing stages, respectively [10]. The mice were injected i.p. with 0.5 ml of PBS alone or containing cisplatin at a dose of 5 mg/kg body weight, 48 h prior to harvesting of DLAF or serum. This protocol was selected on the basis of our previous observation, showing optimum effect of cisplatin after a lapse of 48 h administration [3].

2.4. Isolation of serum

Serum from early (5 days) and late (17 days) DL-bearing mice was obtained following a standard protocol in which the blood was allowed to clot at room temperature for 1 h and then left at 4 °C overnight for clot retraction. Serum was then collected by centrifugation at $1500 \times g$ for 30 min at 4 °C, sterilized by filtration with a 0.45 μm membrane and stored at −20 °C until use [18].

2.5. Preparation of DL cell-free ascitic fluid (DLAF)

DLAF was prepared as described earlier [10]. DL was aspirated by peritoneal lavage after 5 or 17 days following DL transplantation. The peritoneal exudates fluid thus obtained were centrifuged at $200 \times g$ for 10 min at 4 °C. The cell-free supernatant was collected and passed through a 0.22 μm membrane filter and stored at −20 °C until use.

2.6. Morphological evaluation of apoptotic DL cells

Apoptotic cell population was enumerated by a method described earlier [20]. Cell suspension was smeared on a slide and air-dried, fixed in methanol, stained with Wright staining solution and analysed under microscope (Nicon, Japan) at 400× magnification. Apoptotic cells were identified on the basis of morphological features that included contracted cell bodies, condensed, uniformly circumscribed and densely stained chromatin, and membrane bound apoptotic bodies containing one or more nuclear fragments (Fig. 9). The percentage of apoptotic cells was determined by counting more than 300 cells in at least three separate microscopic fields.

2.7. Percent DNA fragmentation

Quantitative determination of DNA fragmentation was carried out following a method given by Sellins and Cohen [20] with slight modifications described previously [3].

2.8. SDS-PAGE and Western immunoblot analysis

Western immunoblot analysis was carried out following methods reported earlier [3]. DL cells were washed with chilled PBS and then lysed in 50 μl of lysis buffer (20 mM Tris–Cl, pH 8.0, 137 mM NaCl, 10% v/v glycerol, 1% v/v Triton X-100, 2 mM EDTA; 1 mM phenylmethylsulfonyl fluoride, 20 μM leupeptin containing aprotinin at 0.15 U ml^{-1}) for 20 min at 4 °C. Protein content in each sample was determined by using standard Bradford method [21]. 30 μg of Triton X-100-solubilized proteins was separated on 10% SDS-polyacrylamide gel at 20 mA. The gel was either stained with Comassie Brilliant Blue (R250) for analysis of proteins bands or processed further for Western immunoblotting. The separated proteins were transferred onto a nitrocellulose membrane (Sartorius, Gottingen Germany) (1.5 h at 150 mA), immunoblotted with antibodies against indicated proteins and probed with a secondary antibody: anti-rabbit IgG conjugated to alkaline phosphatase and detected by a BCIP/NBT solution (Amresco, Solon, Ohio, USA). The blotting membrane was striped and reprobed with anti-(-actin to check equal loading of protein.

2.9. ELISA for detection of cytokine in DLAF and DL serum

A standard ELISA was performed to detect the presence of indicated cytokine in ascitic fluid and serum of tumour-bearing mice or culture supernatant of *in vitro* culture of DL cells following a method described earlier [3]. ELISA for cytokines was compared to standard preparation of the respective cytokines obtained from National Institute for Biological Standards and Control (NIBSC) (Hertfordshire, UK).

2.10. Assay for tumour cell survival

Survival of DL cells was assayed according to a method described earlier [3] with slight modifications. DL cells were seeded (1×10^6 viable cells 200 μl^{-1} ml^{-1}) in a 96 well tissue culture plate in medium alone or containing the serum or DLAF 10% (v/v) and incubated at 37 °C in a CO_2 incubator for 48 h. Cell survival was measured by a standard MTT assay, described below.

2.11. MTT assay

MTT assay was carried out to estimate DL cell proliferation, following a method described by Mosmann [22]. MTT [3-(4,5-dimethylthiazol-2yl)-2,5-diphenyl tetrazolium bromide] was dissolved in PBS at a concentration of 5 mg/ml of MTT solution; 50 μl were added to each well of the culture plate containing 200 μl medium and incubated at 37 °C for 4 h. The medium then was removed carefully, without disturbing the dark blue formazan crystals. 50 μl DMSO, were added to each well and mixed thoroughly to dissolve the formazan crystals. The plates were then read on a microplate reader (Labsystems, Helsinki, Finland) at a wavelength of 540 nm. Readings were presented as optical density (O.D.) at 540 nm.

2.12. Nitrite assay

The concentration of stable nitrite NO^{2-}, the end product from NO generation by effectors macrophages, was determined by the method of Ding et al. with slight modification [10,23] based on the Griess reaction. Culture supernatants were incubated with an equal volume of Griess reagent [1 part of 1% (w/v) sulfanilamide in 2.5% H_3PO_4 plus 1 part of 0.1% (w/v) naphthyl–ethyline–diamine dihydrochloride; two parts being mixed together within 12 h of use and kept chilled] at room temperature for 10 min in a 96-well microtiter plate. The absorbance at