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Cytokine profile of conditioned medium from human tumor cell lines after acute and fractionated doses of gamma radiation and its effect on survival of bystander tumor cells

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ABSTRACT

Cytokines are known to play pivotal roles in cancer initiation, progression and pathogenesis. Accumulating evidences suggest differences in basal and stress-induced cytokine profiles of cancers with diverse origin. However, a comprehensive investigation characterising the cytokine profile of various tumor types after acute and fractionated doses of gamma-irradiation, and its effect on survival of bystander cells is not well known in literature. In the present study, we have evaluated the cytokine secretion profile of human tumor cell lines (HT1080, U373MG, HT29, A549 and MCF-7) either before (basal) or after acute (2, 6 Gy) and fractionated doses (3×2 Gy) of gamma-irradiation in culture medium obtained from these cells by multiplex bead array/ELISA. Moreover, clonogenic assays were performed to evaluate the effect of conditioned medium (CM) on the survival and growth of respective cells. Based on the screening of 28 analytes, our results showed that the basal profiles of these cell lines varied considerably in terms of the number and magnitude of secreted factors, which was minimum in MCF-7. Interestingly, TNF-α, IL-1β, PDGF-AA, TGF-β1, fractalkine, IL-8, VEGF and GCSF were found in CM of all the cell lines. However, secretion of certain cytokines was cell line-specific. Moreover, CM caused increase in clonogenic survival of respective tumor cells (in the order HT1080 > U373MG > HT29 > A549 > MCF-7), which was correlated with the levels of IL-1β, IL-6, IL-8, GMCSF and VEGF in their CM. After irradiation, the levels of most of the cytokines increased markedly in a dose dependent manner. The fold change in cytokine levels was lower in irradiated conditioned medium (ICM) of tumor cells collected after fractionated than respective acute dose, except in MCF-7. Interestingly, amongst these cell lines, the radiation-induced fold increase in cytokine levels was maximum in ICM of A549 cells. Moreover, bystander A549 cells treated with respective ICM showed dose dependent decrease in clonogenic survival. In conclusion, present study revealed the similarities and subtle differences in basal and radiation-induced cytokine profile of different tumor cell lines, and its influence on growth and survival of respective bystander cells. These findings may add a new dimension to our current understanding about role of cytokines in cancer biology.

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1. Introduction

Cytokines are known to influence the process of cancer initiation, pathogenesis and therapeutic outcome of various anticancer treatments [1–4]. Role of cytokines in various aspects of cancer biology like survival, proliferation and motility of malignant cells, cell–cell or cell–matrix interactions, neovascularization, immune-cell infiltration and therapeutic resistance have been established and reviewed [5,6]. The profile of cytokines in tumor microenvironment is highly dynamic and subject to multitude of changes at various stages of tumor development [7–9]. Most of the tumors are known to modulate their cytokine secretion in response to stresses like chemotherapeutic drugs, ionizing radiation, hypoxia [10–14], etc. and hence govern the therapeutic outcome of cancer.

Abbreviations: EGF, epidermal growth factor; FGF-2, fibroblast growth factor-2; Flt3-L, Fms-related tyrosine kinase 3-ligand; GCSF, granulocyte colony stimulating factor; GMCSF, granulocyte monocyte colony stimulating factor; IFN-γ, interferon-γ; IL-1α, interleukin-1α; IL-1β, interleukin-1β; IL-1ra, interleukin-1 receptor antagonist; IL-2, interleukin-2; IL-3, interleukin-3; IL-6, interleukin-6; IL-8, interleukin-8; IL-10, interleukin-10; IL-15, interleukin-15; IL-17, interleukin-17; IP-10, interferon-γ inducible protein-10; MCP-1, monocyte chemotactic protein-1; MIP-1, macrophage inflammatory protein-1; PDGF-AA, platelet derived growth factor-AA; PDGF-BB, platelet derived growth factor-BB; sCD40L, soluble CD40 ligand; sIL-2rα, soluble interleukin-2 receptor α; TGF-α, transforming growth factor-α; TGF-β, transforming growth factor-β; TNF-α, tumor necrosis factor-α; VEGF, vascular endothelial growth factor.

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Moreover, these soluble factors also interact with non-targeted cells surrounding the tumor tissue, and hence, may determine the magnitude of damage to non-targeted tissues via bystander effect [15–17] during therapeutic conditions.

Elevated expression of cytokines is a common phenomenon of cell lines derived from cancer types like melanoma, leukemia, and gastric and ovarian carcinomas [18–21]. Existence of cytokine network has also been studied in biopsy samples and xenograft models, [22] suggesting the diversity in cytokine profiles of various tumor types at mRNA and protein levels. For instance, Hazelbag et al. have shown mRNA expression of TGF-β, IL-4, IL-15 in a majority of cervical cancer cell lines [23]. Whereas, TGF-β and IL-10 mRNA were commonly expressed in primary brain tumors [24]. Most of the ovarian cancer biopsy samples were found positive for IGF-1, IL-6, TNF-α, TGF-α, M-CSF, IL-8 [20]. However, comparative analysis of basal and radiation-induced cytokine profile of tumors with diverse origin and multifunctional etiology is not well documented.

The factors secreted from cells in culture medium include metabolites, cytokines, growth factors, etc., which are known to facilitate the survival of fresh cultures. The phenomenon is known as 'conditioning effect' and the medium as 'conditioned medium (CM)'. Such culture conditions simulate and provide convenient experimental system to study the soluble factors secreted from tumor cells. In the present study, five human tumor cell lines; HT1080 (fibrosarcoma), U373MG (glioblastoma), HT29 (colon carcinoma), A549 (lung adenocarcinoma) and MCF-7 (breast adenocarcinoma) were used to compare their cytokine profiles with or without γ-irradiation. The present study was aimed (i) to evaluate the basal level of common/unique cytokines secreted by different cancer types, (ii) to study the qualitative and quantitative alterations in cytokine profile of tumor cells after acute and fractionated doses of radiation, and (iii) to understand the effect of the cytokine profile of CM/ICM on the survival of respective bystander tumor cells.

2. Materials and methods

2.1. Cell culture

HT1080, U373MG, HT29, A549 and MCF-7 were obtained from National Centre for Cell Sciences, Pune, India and cultured in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% fetal calf serum (FCS) and antibiotics (100 units/ml penicillin and 100 μg/ml streptomycin) at 37 °C in 5% CO_2 atmosphere. Cells were maintained in exponentially growing culture condition and passaged twice a week. Cells were routinely checked for absence of mycoplasma using mycoplasma detection kit (Sigma Chemical Co., USA) as per instructions provided by the manufacturer.

2.2. Collection of conditioned and irradiated conditioned media

Cells (1×10^6) were seeded in 100 mm culture dish (Falcon, USA) in 10 ml complete medium for desired culture period (24 and 48 h). Medium was collected from these cultures, centrifuged at $1500 \times g$ for 10 min, supernatants, (i.e. CM) were separated and stored at −20 °C until use. Plain medium (PM) i.e. complete medium without cells was incubated under same experimental conditions and used as control. To obtain the irradiated conditioned medium (ICM), cells (1×10^6) were seeded for overnight followed by replacement of medium with the fresh one. These cells were γ-irradiated (2 Gy or 6 Gy) under sterile conditions and ICM were collected at 48 h after irradiation following protocol similar to the collection of CM. To irradiate cells with fractionated dose equivalent to 6 Gy, cultures were irradiated with three doses of 2 Gy with the interval of 12 h between successive irradiations (schedule of irradiation and collection of CM and ICM is shown in Fig. 1). The 12 h interval was chosen to match the total time period followed in irradiation schedule of respective acute dose (6 Gy). All irradiation were carried out at room temperature (dose rate: 1 Gy/min) using ^{60}Co gamma teletherapy machine (Bhabhatron-II, Panacea Medical Technologies, Bangalore, India). Wherever required, cultures were trypsinized and cell count was taken using haemocytometer.

2.3. Quantification of growth factors/cytokines

We have used multiplex bead array as a rapid, sensitive and high throughput technique in addition to ELISA to quantify tumor-derived secretable factors (cytokines, cytokine receptor and receptor antagonist) in CM and ICM of tumor cell lines. The dynamic range of multiplex bead array is from 0.1 to 10,000 pg/ml and specific detectability for each analyte is given in Supplementary Table S1. For multiplex bead array, samples were processed for a panel of selected analytes using human cytokine/chemokine assay kit (Millipore) following established protocol, with suitable standards and quality controls (Vimta Lab, Hyderabad, India). In brief, 96 well plate (Millipore, USA) was rinsed with PBS (200 μl/well) under gentle shaking for 10 min. at room temperature, followed by aspiration by vacuum. Standards and samples (CM and ICM; 25 μl) were dispensed per well in triplicates. Equal amount of assay buffer and PM was added to background and blank control wells, respectively. Capture antibody–bead mixture (25 μl) was added to each well and incubated overnight at 4 °C. Subsequently, 25 μl biotinylated detection antibody was added in each well. After 1 h incubation, 25 μl streptavidin–phycoerythrin was added and incubated further for 30 min., at room temperature. Each step was followed by twice washing of wells using 0.05% Tween-20 in PBS (PBST). After the final washing, 150 μl sheath fluid was added per well and read using Luminex based Bioplex-200™ platform (BioRad, USA). The standard curve was plotted and analyte concentration in samples was calculated and expressed in pg/ml.

Out of the analytes mentioned in this study, three analytes (IL-1β, TGF-β1 and EGF) were measured by enzyme-linked immunosorbent assay (ELISA) using BD (IL-1β and TGF-β1), R&D (EGF) ELISA kit following the instructions provided by the manufacturer.

To normalize the analyte concentration with respect to cell number, concentration of a particular analyte was divided by the cell number at the time of CM/ICM collection. The values obtained were further multiplied by 10^6 to obtain analyte concentration per 10^6 cells. The fold change for an analyte after particular dose of radiation was calculated as following: [(Analyte concentration per 10^6 cells in irradiated sample) ÷ (Analyte concentration per 10^6 cells in unirradiated control)].

2.4. Clonogenic assay

The clonogenic assay was performed using standard protocol. In brief, 500 (A549) or 250 (HT1080, U373MG, HT29, MCF-7) cells were seeded in 60 mm dish in 4.5 ml of respective CM (obtained from 1×10^6 cells cultured for 48 h) or ICM and the control PM. After 11 days of incubation, plates were rinsed with PBS and fixed with absolute ethanol followed by staining with crystal violet (1% w/v). The colonies (with >50 cells) were counted using stereomicroscope. The percentage plating efficiency (%PE) was calculated as: (number of colonies obtained ÷ number of cells seeded) × 100. For some experiments, PM and CM were treated with neutralizing antibodies (NAs) against, IL-1β (0.05 μg/ml), IL-8 (1 μg/ml), GMCSF (0.2 μg/ml) and VEGF (0.2 μg/ml) (R&D) for 1 h. The concentrations chosen were two folds of recommended neutralization dose (ND_{50}; as mentioned in technical data sheets). These NA-treated PM and

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