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Enhancement of radionuclide induced cytotoxicity by 2-deoxy-D-glucose in human tumor cell lines

ABSTRACT

The efficacy of targeted radiotherapy can be enhanced by selective delivery of radionuclide to the tumors and/or by differentially enhancing the manifestation of radiation damage in tumors. Our earlier studies have shown that the 2-deoxy-D-glucose (2-DG), an inhibitor of glucose transport and glycolytic ATP production, selectively enhances the cytotoxicity of external beam radiation in tumor cells. Therefore, it is suggested that 2-DG may also enhance the cytotoxic effects of radionuclides selectively in tumor cells, thereby improving the efficacy of radionuclide therapy. In vitro studies on breast carcinoma (MDA-MB-468) and glioma (U-87) cell lines, has been carried out to verify this proposition. Clonogenicity (macrocolony assay), cell proliferation, cytogenetic damage (micronuclei formation) and apoptosis were investigated as parameters of radiation response. Mean inactivation dose D (dose required to reduce the survival from 1 to 0.37), was 48 MBq/ml and 96 MBq/ml for 99 mTc, treated MDA-MB-468 and U-87, respectively. The dose response of growth inhibition, induction of micronuclei formation and apoptosis observed under these conditions, were correlated well with the changes in cell survival. Presence of 2-DG (5 mM) during radionuclide exposure (24 hrs), reduced the survival by nearly 2 folds in MDA-MB-468 (from 48.5 MBq to18.5 MBq) and by 1.6 folds in U-87 cells (from 96 MBq to 66 Mbq). These results clearly show that the presence of 2-DG during radionuclide exposure, significantly enhances the cytotoxicity, by increasing mitotic as well as interphase death. Further studies to understand the mechanisms of radio-sensitization by 2-DG and preclinical studies using tumor-bearing animals, are required for optimizing the treatment schedule.

KEY WORDS: Radionuclide, micronuclei frequency, 2-deoxy-D-Glucose, survival.

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INTRODUCTION

Targeted radiotherapy involves the use of radiolabeled monoclonal antibodies (mAb) or peptidesthat bind to tumor-associated antigens or receptors, uniquely, or are over-expressed in tumor cells or tumor vasculature.[1] This therapy has certain advantages over external beam therapy, as it can selectively deliver radiation doses to the target tissues. However, small yet undesirable delivery of radiation to the non-target tissues, resulting in few immediate and late side effects[2-4] and the inherent radio-resistance of tumors, limit the success of this therapeutic modality. Therefore, approaches that can improve the selective delivery of radionuclide to the tumors and/or differentially enhance the manifestation of radiation damage in tumors, would significantly enhance the efficacy of radionuclide therapy. Ionizing radiation induces a number of DNA and non-DNA damages,[5] causing profound alterations in the gene expression and cell proliferation.[6,7] It is well established, that the cellular processes leading to the repair and fixation of radiation-induced DNA damage (and possibly non-DNA damage as well, require a continuous supply of metabolic energy in the form of ATP[8-11] produced by the respiratory and/or the glycolytic pathways.[4,12,11] Since tumor cells manifest a higher glucose uptake and derive a large part of their energy from the glycolytic pathway, it was postulated that inhibitors of glucose transport and glycolysis could differentially enhance radiation damage in tumors.[9,4] Subsequently, it was demonstrated that 2-DG, a glucose antimetabolite, selectively inhibits DNA repair in cells with high rates of glycolysis (e. g. cancer cells), thereby enhancing the radiation damage in tumor cells *in vitro and* in animal tumors *in vivo,*[13-17,9,18] following irradiation with low LET, as well as high LET external beam irradiation. On the other hand, a significant decrease in the radiation damage of normal cells has been observed under these conditions, possibly due to reduced damage fixation.[19,20] The basic nature of DNA (and non-DNA] lesions induced by radionuclides and the molecular responses elicited by these lesions, show a great deal of similarity with external beam

irradiation, although the rate of damage induction as well as kinetics of repair/fixation could vary, leading to qualitative as well as quantitative differences in the cellular radio responses. Therefore, it has been proposed that metabolic inhibitors such as 2-DG, could also modify the cellular responses to radionuclide- induced damage, by modulating the repair as well as damage-dependent cell proliferation and cell death processes. To verify this proposition, we have initiated studies to investigate the effects of 2-DG on the cytotoxicity induced by free as well as by radionuclides, conjugated to various specific vectors, that provide different types of radiation (γ and β emitters). In the present communication, wepresent our results on the free 99 mTc (gamma and auger emitter) treated human tumor cell lines.

MATERIALS AND METHODS

Cell culture

Monolayer cultures of human breast adenocarcinoma cells, MDA-MB-468 (kindly provided by Dr. Normando, CIM, Cuba) have been characterized and shown to over- express transcriptionally active mutant p53 protein[21] and human malignant glioma cells. U-87 was obtained from Dr. Stuschke of Radiotherapy Dept, Universitatsklinikam, Essen and carries a wild type TP53, which is not efficiently induced after irradiation.[22] The cultures were maintained at 37°C, in a humidified CO2 incubator (5% CO2, 95% air], in Dulbecco's Modified Eagle's Medium, DMEM (Sigma, USA), supplemented with 10% foetal calf serum (Biological Industries, Israel) 50 units/ml Penicillin, 50 μg/ml Streptomycin sulfate and 2 μg/ml Nystatin. Cells were routinely sub-cultured twice a week, using 0.05% Trypsin (Sigma, USA) in 0.02% EDTA.

Cell survival: 99mTc and 2-DG treated cells were washed twice with HBSS, trypsinized and 100 to 1000 cells were plated in 60-mm petridishes and incubated at 37°C, in 5% CO2 humidified atmosphere (depending on the treatment), for 8-10 days. Colonies were fixed in methanol and stained with 1% Crystal violet. Colonies containing more than 50 cells were counted. To compare the extent of 2-DG induced sensitization or modification among different cell lines, sensitization enhancement ratio- SER, was calculated using formula SERSF 2-DG = D1/D2, where D1 and D2 are the required doses of 99mTc, without and with 2-DG, to obtain a surviving fraction of 0.2. (A value of SERSF
2-DG > 1 means an increase in the damage (sensitivity), while rSF
2-DG <1 means a decrease).

Cell growth

Cells were treated with 99mTc and 2-DG for 24 hrs, trypsinized and replated in fresh growth medium to observe post treatment growth of treated cells. At regular time intervals (24 h, 48 h and 72 h) following the post plating, cells were washed, trypsinized and resuspended in PBS. The cells were enumerated using a haemocytometer.

Cytogenetic damage

Micronuclei expression was studied in the first post- treatment mitosis, by arresting cells at cytokinesis using Cytochalacin B. Treated cells were grown with Cytochalacin B[23] (3 μg/ml) for 24 h, trypsinized and fixed in methanol: acetic acid (3: 1) for at least 2-4 hours.

Air dried cells were stained with Hoechst-33258 at a final concentration of 10 μg/ml, in a buffer containing 50 mM sodium phosphate buffer, 10 mM Citric acid and 0.5% Tween-20 detergent. The binucleated cells with micronuclei, were scored with the help of a fluorescence microscope, using UV excitation. A minimum of 500 cells each from duplicate slides, were scored per group. SER
(SER2-DG) was calculated using formula: SERMN
2-DG= (MNF) 99 m
Tc+2-DG / (MNF) 99 mTc, where
(MNF) 99mTc
+2-DG is the % micronuclei frequency in the 99 mTc+2-DG treated cells and (MNF) 99 mTc is the % micronuclei frequency in 99 mTc- treated cells (A value of SERMN 2-DG > 1 means an increase in the damage (sensitization), while SERMN 2-DG <1 means a decrease).

Apoptosis

Morphological analysis

Apoptotic cells were analyzed, based on characteristic morphological changes in the nuclei, associated with this mode of death.[24] Acetic methanol fixed cells were stained with the fluorochrome, Hoechst-258 (10 μM/ml), as described above. Cells were examined under similar ways, as described for micronuclei analysis. SER (SER2-DG) was calculated using formula:
SERAPOP
2-DG = (% APOP) 99 m
Tc+2-DG / (% APOP) 99 m
Tc, where (% APOP) 99 m
Tc+2-DG is the % apoptotic cells in the 99 mTc+ 2-DG treated cells and (% APOP) 99m
Tc is the % apoptotic cells in 99 mTc (A value of SERApop
2-DG > 1 mean an increase in the damage (sensitivity), while
SERAPOP
2-DG <1 means a decrease).

Phosphatidylserine externalization

Apoptotic cells were also detected, by labeling externalized phosphatidylserine, using Annexin-VFITC in unfixed cells.[25] For these measurements, cells were harvested, by scrapping immediately after 24 hr exposure to 99 mTc, in the presence or absence of 2-DG and aliquots of 1 x 105 cells resuspended in 100 μl binding buffer (10 mM HEPES (NaOH, pH 7.4; 140 mM NaCl; 2.5 mM $CaCl_2$). Annexin-V-FITC (5 μl) and 10 μl of PI (50 μg/ml) were added. After 15 min at room temperature, 400 μl of binding buffer was added to each sample and analyzed by flow cytometry.

The percentages of annexin-V +ve and –ve cells, were

estimated by applying appropriate gates.

Statistical analysis

Cell survival and cytogenetic damage, as well as apoptosis data, were obtained from duplicates or triplicates of three independent experiments. Student's paired t-test was used to examine the significance of differences in the mean values between different groups. *P* value of 0.05 or less was considered statistically significant.

RESULTS

Cell survival

Cell survival was studied as a function of the cumulative dose, by varying the volumic activity, as well as exposure time. 99 mTc- treated MDA-MB-468 cells with a mean inactivation dose (D) of 48 MBq/ml, were more sensitive than U-87 with D value of 96 MBq/ml.

Presence of 2-DG (5 mM) during radionuclide exposure (24 hrs),

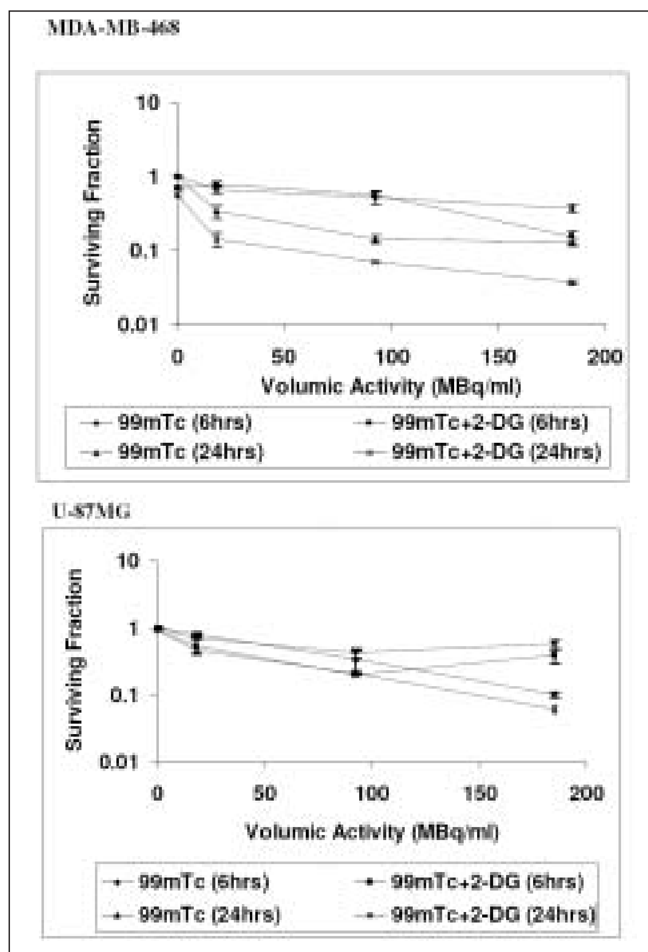

Figure 1: Effects of 2-DG (5 mM) during exposure to varying volumic activity of 99 mTc and treatment time (6 hrs and 24 hrs) on the survival (clonogenic assay] of continuously growing MDA-MB-468 and U-87 cells. Data represents mean (□1SD) from 3 independent experiments

Figure 2: Growth inhibition induced by 99 mTc (92.5 MBq/ml) and 2-DG during and after exposure in continuously growing MDA-MB-468 cells and U-87 cells. Figure 2A and 2B represents growth during exposure and Figure 2C and 2D represents post treatment growth. Data represents mean (□1SD) from 3 independent experiments.

reduced the survival by nearly 2 folds in MDA-MB-468 (from 48.5 MBq to 18.5 MBq) and by 1.6 folds in U-87 cells (from 96 MBq to 66 Mbq). Under these conditions, 2-DG alone was minimally cytotoxic to the U-87 cells, while a 30% cell kill was observed in MDA-MB-468 cells for a 24 hrs exposure, suggesting that the cell kill observed with the combined treatments were clearly supra-addiditive, implying sensitization to 99mTc. However, presence of 2-DG during a 6hrs exposure to 99mTc, enhanced the cytotoxicity by 2.4 fold only at the higher dose, in MDA-MB-468 cells (*P*_0.03), while 1.8 folds enhancement at 92.5 and 185MBq doses, was observed in U-87 cells (*P*_0.0026 and 0.03 respectively).

Cell proliferation

Exposure of MDA-MB-468 and U-87 cells, to a volumic activity of 92.5 MBq/ml, induced both cytostatic as well as cytotoxic effects, resulting in a significant loss in the cell number observed at 24 h of exposure [Figure 2A, 2B]. Presence of 2-DG under these conditions, significantly enhanced both these effects, resulting in nearly 70% reduction in the number of viable cells at 24 h, in both the cell lines [Figure 2A, 2B]. Under these conditions, 2-DG had no significant effect on the growth of un-irradiated U-87 cells, while 40% reduction in cell number was observed in MDA-MB-468 cells, similar to earlier observations.[26] During enumeration of cell number, large amount of cell debris was observed in the treated groups, particularly following the combined treatment. To assess the possible necrotic mode of cell death, besides apoptosis, caused by 99 mTc and 2-DG, we performed phosphatidylserine externalization assay using Annexin-V and PI (propidium iodide) staining, to differentiate apoptotic and necrotic population. The results obtained with Annexin-V assay also confirmed the necrosis and correlated well with the loss of cell number observed immediately after 24 h exposure to 99 mTc and 2-DG, in these cell lines. The re-growth of cells plated following the treatment, was also profoundly inhibited in cells treated with the radionuclide or the combination (radionuclide and 2-DG] (Figure 2C, 2D). However, presence of 2-DG did not have any inhibitory effects on the re-growth of 99mTc treated cells.

Micronuclei Induction

Since mitotic death (related to cytogenetic damage) and apoptosis are the two major modes of cell death, we have investigated the treatment- induced micronuclei formation and

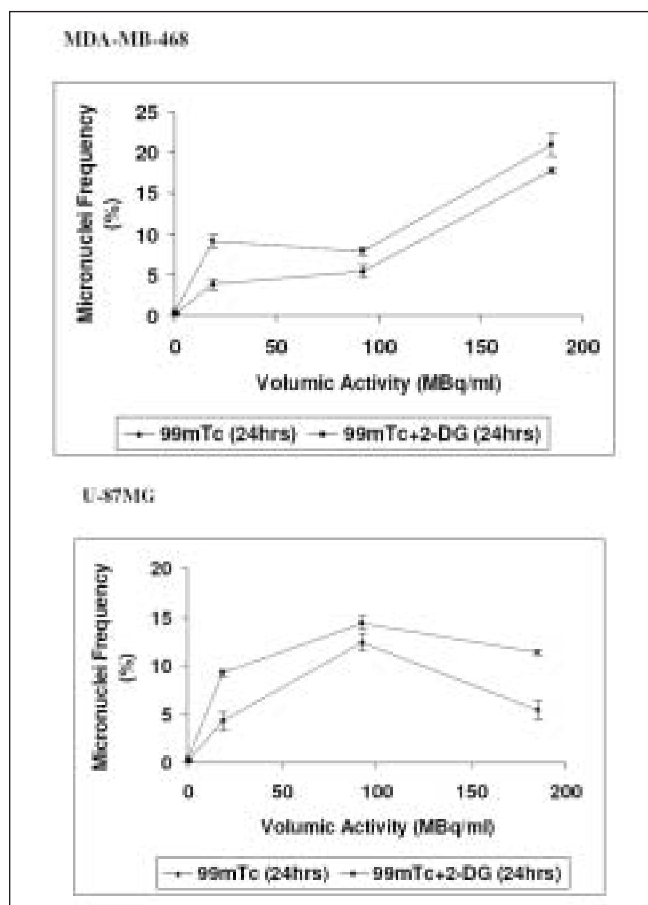

Figure 3: Effects of 2-DG on 99 mTc induced micronuclei expression in asynchronously growing MDAMB-468 and U-87 cell lines. Data represents mean (□1SD) from 3 independent experiments.

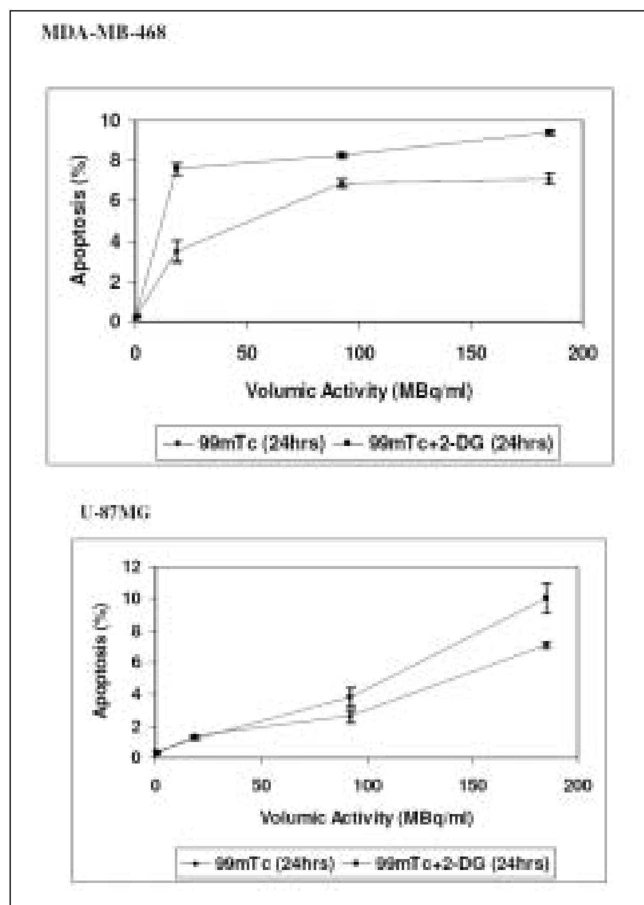

Figure 4: Effects of 2-DG on 99 mTc-induced apoptosis (morphological analysis) in asynchronously growing MDA-MB-468 and U-87 cell line cells. Data represents mean (□1SD) from 3 independent experiments

apoptosis, in both the cell lines. Micronuclei formation was analyzed by arresting the cells in the cytokinesis, using Cytochalacin B.[23] The Cytochalacin B concentration used to achieve more than 90% binucleated cells, was 3 µg/ml, in both the cell lines. Interestingly, a dose- dependent reduction in the percentage of binucleated cells, was observed at 24h exposure to radionuclide in MDA-MB-468 cells, with a decrease of nearly 20%, at 185 MBq/ml. However, a significant change was not observed in U-87 cells at this dose. Micronuclei frequency in binucleated MDA-MB-468 cells increased in a dose dependent manner [Figure 3] and was maximum (21%) at 185 MBq/ml, for an exposure time of 24 h [Figure 3], while in U-87 cells, the maximum micronuclei frequency (11.5%) was observed at 92.5 MBq/ml, which decreased to 4.4% at 185MBq/ml. Presence of 2-DG during radionuclide exposure, enhanced the micronuclei frequency by 2-3 folds, at a volumic activity of 18.5 MBq/ml and nearly 2 folds for 92.5 MBq/ml and no significant effect could be observed at the highest dose (185 MBq/ml] in MDA-MB-468 cells. However, in U-87 cells, an increase was observed at 185 MBq/ml [Figure 3].

Apoptosis

The frequency of apoptotic cells in MDA-MB-468 increased with the increase in volumic activity up to 92.5 MBq/ml and did not

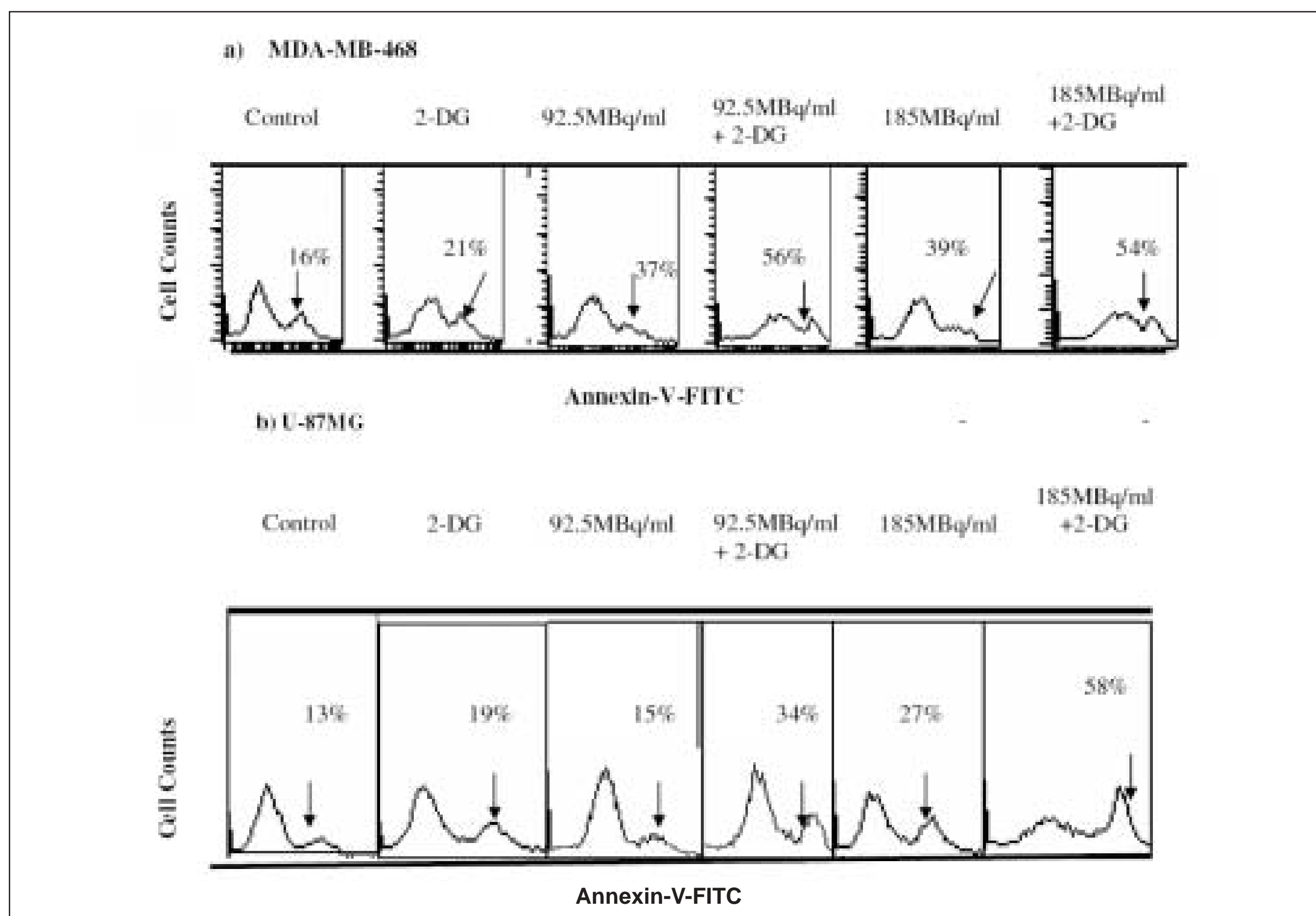

Figure 5: Effects of 2-DG on 99 mTc (92.5 MBq/ml and 185 MBq/ml) induced externalization of phosphatidyl serine (membrane asymmetry) using annexin-V-FITC in asynchronously growing MDA-MB-468 and U-87 cell line cells

Table 1: Sensitization enhancement ratio, SER (SER2-DG) showing the effects of 2-DG on ^{99m}Tc induced damage (cell survival, micronuclei frequency and apoptosis) for isoeffects (in case of survival) and at different volumic activities of ^{99m}Tc (for MN and apoptosis) in MDAMB-468 and U-87 cell lines. SER value <1 indicates sensitization

Parameters	Dose (MBq/ml)	MDA-MB-468	U-87MG
Survival (SF=0.2)		3.7 (SD±0.073)	1.6 (SD±0.077)
	18.5	3.20 (SD±0.01)	1.75 (SD±0.028)
	92.5	1.80 (SD±0.01)	1.30 (SD±0.028)
Micronuclei frequency	185	1.10 (SD±0.01)	2.65 (SD±0.028)
	18.5	2.50 (SD±0.03)	0.95 (SD±0.017)
	92.5	1.20 (SD±0.03)	1.70 (SD±0.017)
Apoptosis	185	1.30 (SD±0.03)	1.50 (SD±0.017)

change further, when the activity was increased to 185 MBq/ml [Figure 4], while a gradual increase was observed up to 185 MBq/ml in U-87 cells.

Presence of 2-DG during exposure to radionuclide, showed dose-dependent increase in the frequency of apoptotic cells, with maximum increase of nearly three folds at 18.5 MBq/ml in MDAMB-468 cells. On the other hand, in U-87 cells, induction of apoptosis was significantly higher (40%), at higher dose 185 MBq/ml [Figure 4]. Under the present experimental conditions, 2-DG alone, did not induce apoptosis in both the cell lines [Figure 4].

Phosphatidylserine externalization

Membrane asymmetry associated with apoptosis, indicated by phosphatidylserine externalization, was assayed using Annexin-V-FITC binding. The fraction of Annexin-V +ve cells at 24 hr exposure to 92.5MBq.ml and 185MBq/ml, was 37% (15% Annexin-V +ve cells + 22% PI +ve cells) and 39% (25% Annexin-V +ve cells + 15% PI +ve cells) in MDAMB-468, while in case of U-87 cells the fractions of Annexin-V +ve cells were 15% (11% Annexin-V +ve cells + 04% PI +ve cells) and 27% (12% Annexin-V +ve cells + 15% PI +ve cells), respectively. Presence of 2-DG during exposure to radionuclide, significantly enhanced the fraction of Annexin-V and PI +ve cells by 2 fold, in both the cell lines [Figure 5].

DISCUSSION

In the present studies, cellular radiation responses to damage caused by a radionuclide 99mTc (gamma rays and auger electrons), as well as modification of these responses by 2-DG, have been investigated in two human tumor cell lines. Results clearly show that the presence of 2-DG, an inhibitor of glycolytic ATP production,[18,22,27] during exposure to 99mTc, significantly enhances the cytotoxic effects of the radionuclide, by increasing the radionuclide- induced cytogenetic damage and apoptosis [Figure 3 and 4]. Nearly 1.5 folds (U-87) and three folds (MDA-MB-468) increase in cell death, observed in these cell lines due to enhanced cytogenetic damage (micronuclei expression) (SER value up to 3.2), linked mitotic death and interphase death (apoptosis) (SER value up to 2.50). [Table 1] clearly shows that 2-DG can enhance the cytotoxic effects of radionuclide (99mTc). However, quantitative differences were evident in the extent of sensitization [Table 1] between the two cell lines, which could arise due to differences in the treatment induced, modification of different cell death pathways regulated by multiple gene products including p53, as well as differences in the degree of glycolysis between the two cell lines. Indeed, a positive correlation between the endogenous level of glycolysis and 2-DG-induced radiosensitization has been demonstrated, besides significant differences observed in the degree of glycolysis among different tumor cell lines.[15] Although a correlation between p53 status and radiosensitization by 2-DG has not been found so far,[15] it is pertinent to note that U-87 has a wild- type p53 status,[22] while the MDA-MB-468 carried a mutated p53 gene.[21] Induction of fewer lesions at low radiation doses, often result in insignificant changes in cell survival (shoulder region), whereas saturation of repair processes at higher doses leads to enhanced cell death. Quantitative differences observed in the extent of sensitization by 2-DG to 99mTc- induced cell death, at different doses in MDA-MB-cells, could partly arise on account of these reasons and which may differ from cell to cell. These observations are similar to our earlier results, on the radio-modifying effects of 2-DG, following external beam irradiation with 60Co gamma rays, in several human and murine tumor cell lines,[16-18,22] where inhibition of DNA repair and cellular recovery processes have been demonstrated.[4,17,22,28,29]

It is well known that as the dose rate of irradiation decreases, cell survival increases, which is predominantly due to the repair of radiation damage in the moderate dose rate range (from 200 cGy/min to 1cGy/min).[30] 99mTc is a gamma ray and auger electron emitting radionuclide and is homogeneously distributed in various sub cellular sites with 48% in nucleus, 17% in microsomes, 20% in mitochondria and 15% in the membrane, so that it can deliver continuously decreasing dose rates as a function of time, due to disintegration of the radionuclide, uniformly throughout the cells.[31] Therefore, varying the volumic activity as well as exposure time, actually represents cumulative dose effects of continuously decreasing dose rate. Nearly 60% cell kill was observed with a 24 hexposure at 18.5 MBq/ml of the radionuclide in MDA-MB-468 cells [Figure 1] and the value of the surviving fraction (0.33) was similar to the SF observed at 5 Gy, at a dose rate of 0.076 Gy/min, delivered from an 60 Co source of external beam irradiation [data not shown]. At this dose rate, repair during irradiation is the predominant cause of the enhanced survival, as compared to high dose rate (2 Gy/min) irradiation.[30] Since it is reasonable to expect that the repair of DNA damage occurs during exposure of cells to radionuclide [24h], inhibition of repair and/or recovery processes, is expected to enhance cell death. Indeed, inhibition of DNA repair and cellular recovery processes by 2-DG, following damage caused by external beam irradiation, has been reported.[17,22,28,29,32]

Under similar conditions, 2-DG alone had minimal cytotoxicity in U-87 cells, whereas a growth-inhibitory effect resulting in 30-40% reduction in cell number, was observed in MDA-MB-468 for 24 hr exposure, similar to the earlier results reported in this cell line.[23] Variation in the 2-DG- induced cytotoxicity has been previously reported and is related to the complexicity of uptake in irradiated cells and to the levels of pro-apoptotic or anti apoptotic proteins.[33] However, alterations in other post-irradiation responses viz. cell cycle perturbation and apoptosis, that influence survival, also contributes to the cytotoxicity. An alternative explanation for the enhanced radionuclide- induced cell death by 2-DG, could also be the redistribution of the radionuclide in the intracellular milieu, due to altered metabolic

state, induced by 2-DG, as has been shown for 64Cu-ATSM in EMT-6 rat breast tumors.[34] However the uptake and intracellular distribution of radionuclide used for current study (99mTc), is known to be neither energy- dependent nor carrier-mediated.[35]

Mitotic (cytogenetic damage) and interphase death (apoptosis), are two predominant modes of cell Death, that contribute to the loss of survival. Mitotic death is linked to the cytogenetic damage, expressed as chromosomal aberrations in the metaphase and manifest in the form of micronuclei-formation in the post mitotic daughter cells, which arise from the residual DNA damage, following induction and repair of DNA lesions. On the other hand, apoptosis is induced by both, membrane as well as DNA damage, which are observed as early as few hours in certain cell systems[36,37] and after many hours or days in epithelial tumor cells.[24] It is also suggested that mitotically dead cells undergo apoptosis as a secondary response, often referred to as delayed apoptosis and observed 1-2 days after irradiation.[7,38,39] The surviving fraction of 0.34 and 0.1 observed following exposure to 95.2MBq/ml and 182.5 MBq/ml of 99mTc in U-87 cells, is comparable to an absorbed dose of 2Gy and 5Gy of external beam gamma rays, at 0.076Gy/min dose rate, where mitotic death linked to cytogenetic damage in the form of micronuclei expression (20-25%), is the primary cause of cell death, with minimal ($<$ 5%) delayed secondary apoptosis.[7] Interestinglyhowever, the dose (activity) dependent changes in the micronuclei expression in U-87 cells, showed a decrease at 182.5MBq/ml, as compared to 92.5MBq/ml [Figure 4]. This was accompanied by a concomitant increase in the fraction of apoptotic cells, implying a possible quantitative difference in the mitotic vs. apoptotic death. The lack of correlation between micronuclei frequency and apoptosis to the loss of clonogenecity, suggests the contribution of other modes of cell death like necrosis and non apoptotic delayed lethal mutations, as suggested by Little et al.[40,41] However, significant differences could not be observed in the modification of these two responses viz. apoptosis and mitotic death by 2-DG, suggesting that the primary effects of 2-DG could be the modification of the residual DNA (and possibly non DNA) damage, on account of inhibition of repair processes.

Indeed both DNA and non DNA damages can induce apoptosis, that depend on several gene products, which facilitate the progression of apoptosis through p53 dependent and independent mechanisms,[42] involving caspase- dependent and independent pathways. Therefore, the possibilities of 2-DG-induced alterations in the signalling mechanisms, could also be partly responsible for the enhanced apoptosis observed. Induction and expression of several genes related to various signaling and metabolic pathways have been recently reported, following the exposure of cells to radiation and 2-DG.[43]

In RIT, the local tumor control achieved, is due to contributions from the radionuclide and/or toxicity compound associated with the vectors. Therefore, radio-modifiers which are able to enhance the manifestations of radionuclide- induced damages, can further enhance the efficacy of radionuclide therapy. Results of the present studies clearly showed that presence of 2-DG during radionuclide exposure, significantly enhances the cellular damages and imply that 2-DG can enhance the efficacy of radionuclide therapy. Preclinical studies in tumor-bearing animals, besides investigations on the effects of the combined treatment (radionuclide + 2-DG) on the damage to normal cells and tissues, are required before contemplating clinical studies.

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