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Optimizing Treatment of brain Tumours: Effects of Lonidamine and 5-bromo-2-deoxy-Uridine on radiation response of human glioma cells .

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Abstract

Effects of Lonidamine on proliferation and radiation response of human glioma (BMG-1) cells grown as monolayer were studied. Lonidamine at a concentration of 20μ g/ml had no effect on cell growth but significantly inhibited proliferation at 30 and 50μ g/ml. Lonidamine and BrdU did not induce damage in exponentially growing un-irradiated cells. Pre-irradiation presence of BrdU (0.8μ M, 24 hr) significantly increased gamma-ray (2 Gy) induced micronuclei formation. Post- irradiation incubation of cells under sub-optimal growth conditions (DMEM + 1% serum, 2 hr) significantly reduced micronuclei formation. Lonidamine (20μ g/ml, 2 hr) significantly increased radiation damage in absence as well as presence of BrdU. Lonidamine (10, 20μ g/ml; 2, 4 hr) also significantly decreased survival of plateau phase cells after gamma rays (2, 4 Gy). These observations suggest that 1) Radiation induced DNA damage in BrdU sensitized cells are partly repairable. 2) Micronuclei formation is an important mechanism in radiomodification by lonidamine. 3) Lonidamine could decrease the BrdU doses required for radiosensitization of proliferating brain tumour cells, reducing thereby the toxic side effects of BrdU-radiation therapy.

Key words -
Lonidamine,
Bromo-2-deoxy-Urindine,
Radiomodification,
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Proliferation response

Malignant gliomas are virtually incurable; with average survival of patients, with high grade astrocytomas, being less than a year. Post-Operative radiotherapy - alone, or in combination with chemo therapy - leads to only a slight increase in patient survival [1], [2]. Particulate radiations, for example, fast neutrons, have also not been effective in increasing patient survival [3].

Halopyrimidines, such as 5-bromo-2-deoxy-uridine (BrdU) have been used as radiosensitizers in clinical trials on brain tumours [4]. The rationale for use of these thymidine analogues is that when present during DNA synthesis, they get incorporated into DNA, leading to increased radio-sensitivity of proliferating cells [5]; whereas the surrounding normal tissues, with little proliferative activity, would not be sensitized. However, toxic side effects, such as myelosuppression have been observed following BrdU administration [6]. Moreover, non-cycling cells would not be sensitized by BrdU due to lack of incorporation.

In vitro studies have shown that substitution of thymidine by these halopyrimidines leads to an increase in radiation induced single and double strand breaks in DNA, majority of which are repairable [7]. Expression of radiation induced lesions in BrdU sensitized cells could, therefore, be increased by inhibiting repair processes.

Lonidamine [1-2, 4-chlorobenzyl-1H-indazole-3-carboxylic acid] has been observed to radiosensitize Meth-A and radiation induced fibrosarcomas in mice [8]. It has also been reported to inhibit the repair of potentially lethal cellular damage (PLD) in plateau phase transformed mammalian and human cancer cells [9], [10]. These observations suggest that lonidamine could increase the manifestation of damage in non cycling tumour cells.

We have recently initiated studies on the effect of Lonidamine on proliferation and radiation response of human glioma cells. The effects of Lonidamine on BrdU sensitized radiation damage in proliferating cells were investigated. No information on radiation induced sub-cellular (cytogenetic or DNA) damage in Lonidamine treated cells is available. Micronuclei represent residual DNA and chromosomal damage in irradiated, proliferating, post-mitotic cells [11]. Micronuclei formation has been observed to correlate with reproductive cell death [12]; and used to study radiomodifying effects of potential chemical adjuvants in cancer therapy [13]. Micronuclei formation was, therefore, used as index of radiation damage in proliferating cells.

Materials and Methods

Cell cultures

A cell line (BMG-1) derived from a mixed glioma, and grown as monolayer in our laboratory was used in these studies. Cultures were grown at 37° C in 25 cm^2 plastic culture flasks (Nune, Roskilde, Denmark) with an initial inoculum of 2×10^5 cells in 6 ml growth medium. Growth medium consisted of Dulbecco's modified minimum essential medium (DMEM, HiMedia, Bombay, India) supplemented with 5% bovine serum and antibiotics (50 units/ml penicillin, 35µ g/ml streptomycin and 2.2µ ml nystatin) . Doubling time of cells during exponential growth under these conditions was 16-20 hr. Cultures had a lag phase of 20-25 hr, entered plateau phase after 4 days, and were sub-cultivated after 5 days. Experimental cultures were grown in plastic petri dishes (55 mm, Laxbro, Poona, India) in 5% CO2 atmosphere, with an initial inoculum of 1.2×10^5 cells in 4 ml growth medium.

Lonidamine

Lonidamine (F. Angelini Research Institute, Rome, Italy) was dissolved in DMSO (10 mg/ml) and sterilized by millipore filteration. This stock solution was stored at -15° C. Appropriate dilutions were made in culture medium.

Treatment

Effects of Lonidamine treatment were studied on the following parameters.

a)

Proliferation response:

Two days after setting up of cultures, the growth medium was removed, cells were washed, and fresh medium containing Lonidamine (20, 30 or 50μ g/ml) was added. Control cultures had 0.5% DMSO (equal to the amount present in 50μ g/ml Lonidamine). Cultures were maintained at 37° C, upto four days after addition of Lonidamine, cultures were trypsinized (0.1% trypsin for 2 min, at 37° C) and cell counts were made after staining with trypsin blue.

b)

Radiation damage in proliferating cells:

BrdU (0.8μ M final concentration) was added 48 hr after inoculation of cells. Cultures were washed after 24 hr of growth in presence of BrdU, and incubated in 2 ml of growth or liquid holding medium (DMEM with 1% serum); with or without Londamine (20μ g/ml final concentration). Cultures were irradiated with 60-CO-gamma-rays (2 or 4 Gy), using theratron 780C (Atomic Energy of Canada Ltd; dose rate 1 Gy/ min, 35 × 35 cms, f.s.d. 80 cms). Cultures were incubated at 37° C for 2 hr, washed to remove Lonidamine and were subsequently grown in Lonidamine free growth medium. Cells were released by trypsinization (0.1% trypsin for 2 min at 37° C), 18, 24, 30 or 45 hr after irradiation . After fixing in Methonol-Acetic acid (3:1), air dried slides were stained with 5μ M diamino-2-phenyl-indole hydrochloride (DAPI, Serva, Heidelberg, Germany) - a DNA specific florochrome) in phosphate buffer (pH 7.4) containing Tween-20. Micronuclei were scored according to criteria of Countryman and Heddle [11], with slight modifications.

Atleast, 2000 cells were scanned from each culture for presence/absence of micronuclei at 400X under a fluorescent microscope. Treatment induced frequency of cells with micronuclei (M-fraction) in the irradiated cultures was calculated after subtracting the values obtained from the corresponding unirradiated control cultures.

c)

Radiation damage in plateau phase cells:

Cultures in plateau phase were washed and incubated in 2 ml DMEM alone, or with 1% serum, with Lonidamine (10, 20μ g/ml) . Irradiation (2 and 4 Gy) was carried out 20-30 min later and cultures were incubated at 37° C. Two or four hr later, cells were trypsinized, and replated at low density - 1000-3000 cells per petri dish (60 mm, Torsons, Calcutta, India) - in growth medium. After 7 days incubation at 37° C, colonies were washed in phosphate buffered saline, fixed in methanol and stained with Giemsa's stain (10%), Colonies were counted under a colony counter; and those colonies with at least 50 cells were scored as survivors.

Results

Effects of Lonidamine on the growth of glioma cells are shown in Figure 1. Presence of Lonidamine at a concentration of 20μ g/ml did not affect proliferation response when observed 2 and 4 days after addition. Cell growth was partly (~30%) inhibited at 30μ g/ml. However, the inhibition was nearly complete (80-90%) at the highest (50μ g/ml) concentration. Quantitatively similar effects were

observed 1 and 3 days after addition of drug (data not shown).

Proliferation response of exponentially growing human glioma cells as a function of Lonidamine concentration: A) 20μg/ml, B) 30μg/ml, C) 50μg/ml. Lonidamine was present continuously for a) 2 days b) 4 days after which the cell counts were made. Values represent Mean ± s.d. of 6-10 cultures.

Frequencies of cells with micronuclei (M-fraction values in the unirradiated culture was app. 1%. Treatment with BrdU or Lonidamine, alone or in combination, did not increase micronuclei in the absence of radiation (data not shown) . Similar effects of radio modifiers have been observed earlier [13]. Pre-irradiation incorporation of BrdU (0.8μ M, 24 hr) significantly increased micronuclei formation (Figure 2b), as compared to irradiation alone (Figure 2a) . Post-irradiation incubation of cells, for 2 hr in DMEM + 1%, before addition of growth medium, significantly reduced M-fraction values .

Effects of post-irradiation incubation of BMG-1 cells under sub-optimal growth conditions (DMEM + 1% serum), instead of growth medium for 2 hr, on radition induced frequencies of cells with micronuclei (M-fraction). Values represent mean ± s.d. of 3-4 cultures. Atleast 2, 000 cell cultures were analysed for presence or absence of micronuclei, by two observers.

Effects of BrdU and Lonidamine treatments on radiation induced frequencies of cells with micronuclei (M-fractions) in exponentially growing human glioma (BMG-1) cells. After removing BrdU, cultures were incubated in DMEM + 1% serum (2hr) with or without Lonidamine. Damage was assayed as a function of post-irradiation time. Values represent mean=s.d. of 3-4 cultures. Atleast 2, 000 cells were analysed from each culture by two observers.

Treatment with Lonidamine (20μ g/ml; 2 hr in DMEM + 1% serum) significantly increased micronuclei formation in gamma irradiated cells. In the BrdU treated cultures also, Lonidamine further enhanced radiation damage (Figure 3).

Table I - Effects of Lonidamine (2 hr, in DMEM + 1% serum) and 60-Co-gamma rays on survival of plateau phase human glioma cells

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Values represent Mean +s.d. of 4 to 6 cultures

Table II - Effects of Lonidamine and 60-co-gamma rays on survival of plateau phase human glioma cells

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After irradiation, cells were incubated in DMEM alone; with or without Lonidamine for 4 hr. before replating) Values represent Mean ± s.d. of 4-6 cultures

Preliminary observations on the effects of Lonidamine on the survival of BMG-1 cells in the absence and presence of gamma-rays, (2 and 4 Gy) are shown in Tables I and II. In the absence of gamma-rays, Lonidamine did not significantly influence cell survival under most experimental conditions (20μ g/ml, 2 hr in DMEM + 1% serum; 10μ g/ml, 4 hr in DMEM without serum) . When 20μ g/ml Lonidamine was present for 4 hr in DMEM alone, the cell survival was decreased by 13%. However, combination of Lonidamine (20μ g/ml, 2 hr in DMEM + 1% serum) with gamma-rays (2 Gy), decreased the cell survival to 58% of irradiation alone. When the recovery from damage was allowed for 4, instead of 2

hr (before replating, Table II), the cell survival significantly increased (from 48.8% to 67.3%), though the radiation dose was higher (4 Gy). This indicates that these cells are proficient in the repair of potentially lethal damage (PLD) . However, Lonidamine (10μ g/ml, 4 hr, in DMEM alone) decreased the cell survival by 50%. Higher concentration (20μ g/ml) of Lonidamine had no significant further effect; indicating that nutritional conditions (for example serum) and duration of Lonidamine treatment influence cellular response to radiation damage.

Discussion

Earlier studies had shown that Lonidamine at a concentration of 50μ g/ml, (24 hr) did not inhibit proliferation response of chinese hamster (HA-1) cells [9]. There was no inhibition of the growth of HeLa cells at 25μ g/ml, 60% inhibition at 50μ g/ml; and nearly complete inhibition at 100μ g/ml concentration of Lonidamine in 4 days [14]. Therefore, data presented here (Figure 1) demonstrate that BMG-1 cells are more susceptible to cytostatic effects of Lonidamine .

Increase in gamma-induced micronuclei formation by Lonidamine (Figure 3) shows that damage in proliferating tumour cells could also be increased by Lonidamine. These observations (reported for the first time) suggest that cytogenetic damage is an important mechanism in radiomodification by Lonidamine. This could be due to the inhibition of post-irradiation repair processes by Lonidamine.

Earlier studies have shown nearly complete (90-100%) inhibition of PLD repair by Lonidamine (10μ g/ml 3-7 hr in HBSS) after very high doses (8-12 Gy) of Xrays [9], [15]. Data presented in Tables I and II show that Lonidamine could inhibit the repair of PLD in tumour cells even after relatively lower radiation doses (used per fraction in radiation therapy protocols).

The radiomodifying effects of Lonidamine have been explained on the basis of its effects on cellular energy metabolism. It has been reported to inhibit oxidative metabolism in normal and cancer cells. However, it stimulates glycolysis in normal cells while inhibiting glycolysis in tumour cells. These differential effects have been attributed to the mitochondrially bound hexokinase; present in tumours, but absent in normal cells [16], [17].

Implications for improving treatment of brain tumours

Lonidamine (oral administration) has been tested in phase I/II clinical trials on patients with brain and also other types of tumours [18], [19], [20]. The toxicities induced by Lonidamine administration, such as myalgias, mild hairloss, somnolence, and testicular pain in males, are not severe [20], [21]. Peak plasma levels upto 35μ g/ml one to two hr after administration of Lonidamine have been reported in these studies [20]. Present studies (Figure 3) show, that Lonidamine concentrations of 20μ g/ml, maintained for a short time (~2hr) after irradiation, could enhance damage in proliferating tumour cells. Lonidamine has been reported earlier [9] to radiosensitize murine tumours, without increasing side effects like radiation induced skin lesions.

Present observations in human glioma cells (Figure 2) confirm earlier studies in other systems [7], [22], that radiation induced lesions in BrdU sensitized proliferating cells are atleast partly repairable. Therefore, the slow proliferating cell populations would be poorly sensitized by BrdU, due to manifestation of repair processes [23]. Enhancement of radiation damage by Lonidamine, under sub-optimal growth condition, observed in human glioma cells (Figure 2) suggests that Lonidamine could increase radiation damage in slow proliferating brain tumour cells. Combination of BrdU and Lonidamine treatments could, therefore, significantly increase damage, as compared to radiation alone.

Therefore, present observations suggest that clinically feasible doses of Lonidamine could optimize BrdU-radiation therapy of cancer by decreasing the effective BrdU doses required for radiosensitization of proliferating tumour cells; reducing thereby its toxic side effects. In addition. these data (Tables I and II) suggest that Lonidamine could also enhance damage in the non-cycling tumour cell populations with different nutritional conditions; which would not be radiosensitized due to lack of BrdU incorporation. These concentration ranges ((20μ g/mI) of Lonidamine may not produce cytostatic effects in proliferating tissues outside the radiation field (Figure 1).

2-deoxy-D-glucose, another inhibitor of glycolytic energy metabolism was observed to enhance radiation damage in the plateau phase BMG-1 cells only under conditions of respiratory impairment but not in the euoxic cells [13]. Therefore, Lonidamine could possibly be more effective in increasing radiation damage in tumour cell populations, as compared to 2-DG. Moreover, Lonidamine could be more readily acceptable in radiotherapy protocols, as it has been widely tested in clinical studies, including brain tumours. About 90% of brain tumours after surgery are referred for radiotherapy (B.S. Das, Personal communication, 1990). Therefore, it is imperative to develop more effective treatment modalities for these tumours.

Detailed investigations on the effects of Lonidamine on normal and brain tumour cells as well as the mechanisms underlying these effects are envisaged in further studies.

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