Abstract. Background: Lymphomas growing in the central nervous system exhibit resistance to radiotherapy compared to lymphomas of the lymph nodes. Because astrocytes have been shown to reduce radiation-induced neuronal toxicity, this study hypothesized that astrocytes might protect lymphoma cells from radiation-induced cell killing. Materials and Methods: A human lymphoma cell line, H9, and normal human astrocytes were grown in culture, exposed to radiation and assessed for cell viability, radiation sensitivity, glutathione content, induction of apoptosis and cell-cycle distribution. Results: Cell survival assays demonstrated that H9 cells growing in an astrocyte-monolayer and also in an astrocyte-conditioned medium displayed radioresistance compared with H9 cells growing under standard conditions. The radioresistance correlated with accumulation of H9 cells in the G2 phase of the cell cycle, suppression of radiation-induced apoptosis and coincided with a moderate increase in glutathione. Conclusion: The findings of this study suggest that astrocytes may play a role in the radioresistance exhibited by lymphomas of the central nervous system.

Primary central nervous system lymphoma (PCNSL) is a rare form of non-Hodgkin lymphoma that is restricted to the central nervous system (CNS) and accounts for approximately 1-3% of all CNS malignancies (1, 2). The vast majority of PCNSLs are B-cell lymphomas with T-cell variants accounting for only 4% of the cases (3). Patients affected by PCNSL can be divided into two categories: immune-compromised and immune-competent. The former category mostly includes patients affected by human immunodeficiency syndrome (HIV) but also patients that receive immunosuppressive medication after organ transplantation and patients with congenital immunodeficiency syndromes. In the setting of immune-compromised patients, PCNSL commonly arises from Epstein-Barr virus infection of lymphocytes. No known etiology exists for immune-competent patients. According to data from Surveillance, Epidemiology and End Results registries, the age-adjusted incidence increased by three-fold from the 1970s to the 1990s. This increase can most likely be attributed to the HIV epidemic, new imaging technologies that facilitate the diagnosis of this disease, as well as an increasing number of organ transplantations (2, 4).

The combination of radiation therapy and methotrexate-based chemotherapy is the mainstay for treatment of PCNSL (5). Combined modality therapy is usually delivered with induction chemotherapy followed by whole-brain radiotherapy and, according to a recent review, results in a median overall survival ranging from 15-60 months (2). Although PCNSL is histopathologically identical to the same type of systemic non-Hodgkin lymphoma, PCNSL is known to be more resistant to radiation treatment. The literature indicates that while 40 Gy of radiation achieves more than 90% local control rate of systemic non-Hodgkin lymphomas outside of the CNS (6-13), doses as high as 60 Gy fail to achieve local control in many patients with PCNSL. Nelson et al. (14) reported that the local control rate of patients with
PCNSL who received a total radiation dose of 60 Gy was approximately 50%. The ideal radiation dose for PCNSL is controversial. Based on studies mostly conducted before combined modality therapy was established, it has been recommended that it should be somewhere in the range of 40-50 Gy (14-16). Patients receiving less than 40 Gy had poorer outcomes in terms of survival and those receiving more than 50 Gy had poorer outcomes in terms of neurotoxicity (15-17).

Besides local tumor control, neurologic toxicity after chemo-radiation is important in PCNSL. Most important is the delayed neurotoxicity that can include symptoms such as progressive behavioral changes, ataxia and incontinence in elderly patients. Magnetic resonance imaging scans may show progressive leukoencephalopathy (2). On the biological level, there is increasing evidence that astrocytes may be an important component of the radiation response of normal brain tissue. In a study published nearly a decade ago, it was demonstrated that astrocytes protect against radiation-induced neuronal toxicity in vitro (18). In this study, rat cortical neurons were significantly less sensitive to radiation in co-culture with astrocytes or in the presence of astrocyte-conditioned medium (ACM). By comparing ACM that was fractionated into high (>10 kDa) and low (<10 kDa) molecular weight fractions, the authors concluded that radioprotection seems to be mediated by a soluble protein(s) of molecular weight >10 kDa. Based on these findings, the present study hypothesized that astrocytes may also protect human lymphoma cells from radiation-induced toxicity, which might be a factor contributing to the relative radioresistance of PCNSL.

Materials and Methods

Cell culture. H9 (human T-cell lymphoma line) cells were maintained in RPMI-1640 supplemented with 10% fetal bovine serum (FBS), 1% L-glutamine, and 10,000 U/ml of penicillin-streptomycin. Normal human astrocytes (Clonetics Astrocyte Cell Systems; Cambrex Bio Science, Walkersville, MD, USA) were cultivated in AGM Basal Medium (ABM) supplemented with Single Quot additives (both from Cambrex Bio Science).

Generation of conditioned medium. After astrocytes (growing in ABM) became confluent, ABM was completely removed and exchanged against RPMI-1640 medium supplemented with 10% FBS, 1% L-glutamine, and 10,000 U/ml of penicillin-streptomycin. Normal human astrocytes (Clonetics Astrocyte Cell Systems; Cambrex Bio Science, Walkersville, MD, USA) were cultivated in AGM Basal Medium (ABM) supplemented with Single Quot additives (both from Cambrex Bio Science).

Cell-survival assays. Cell-survival experiments were carried out using a modification of the protocol described by Ehmann et al. (19). To assess radiosensitivity, H9 cells (2×10^5 cells/ml) in 1-cm dishes were treated with 0, 5 and 10 Gy of irradiation (γ-rays, using a ^137Cs source; dose rate, 3.7 Gy/min). Cultures were regularly diluted with fresh medium. Cell numbers were counted every other day using a Coulter counter. The resulting set of growth curves allowed extrapolation of the exponential portion of the post-treatment growth curve back to time 0 to obtain the number of cells that had survived in comparison with the untreated cells.

Limiting-dilution survival assay. A limiting-dilution assay was carried out using a modification of the protocol by Green et al. (20). Co-culture with astrocytes was plated in 96-well plates. After they became confluent and stopped dividing, the medium was removed and a known number of H9 cells in RPMI-1640 medium were seeded; cell number dilutions ranged from 0.1 to 3,200 cells per well with twelve different cell numbers and each cell number group having eight duplicate wells. Exact H9 cell number dilutions were seeded also on top of confluent monolayer astrocytes. H9 cells grown in 96-well plates for 48 h alone or on top of monolayer astrocytes were exposed to graded doses (0, 2.5, 5 and 7.5 Gy) of radiation (γ-rays, using a ^137Cs source, dose rate 3.7 Gy/min).

Conditioned medium. A known number of H9 cells in unconditioned RPMI-1640 medium or in ACM were seeded into 96-well plates. After 48 h, H9 cells were exposed to 5 Gy of radiation.

Construction of survival curves. Fourteen days after radiation, wells that were positive for H9 lymphoma cells were scored. The quantitative data were fitted by a logistic regression to determine the number of cells needed to achieve growth in 50% of the wells at the respective dose level (D50). Survival fractions were calculated as the ratio of the D50 of irradiated cells to the D50 of non-irradiated cells.

Glutathione (GSH) assay. Total cellular GSH levels were measured following the method of Hissin and Hilf (21). Briefly, cells were washed once and then resuspended at 1×10^7 cells/ml in cold lysis buffer consisting of 5% trichloroacetic acid: 1 mM EDTA: 0.1 M HCl (1:1:1, v/v/v). For the measurement of GSH, cell lysate (0.2 ml) was mixed with 3.6 ml of 0.1 M phosphate/5 mM EDTA buffer (pH 8.0) and 0.2 ml of o-phthalaldehyde stock (1 mg/ml in methanol) added. Fluorescence was read at 420 nm with excitation set at 350 nm. This measurement was performed in triplicates for each sample. Standard curves were determined using known quantities of GSH and were used to convert fluorescence readings to GSH concentrations.

Cell-cycle assay. H9 cells growing for 48 h in RPMI-1640 medium or ACM were exposed to a single radiation dose of 5 Gy. Forty-eight hours later, cells were subjected to propidium iodide staining and analyzed by flow cytometry for cell-cycle distribution.

Terminal dUTP nick-end labeling (TUNEL) assay for apoptotic cells. H9 cells were grown in RPMI-1640 medium or ACM for 48 h and then exposed to 2-10 Gy of radiation. Four hours later, cells were subjected to TUNEL assay (Apo-direct Kit; BD Biosciences, San Jose, CA, USA) following the procedure provided by the manufacturer. Cells were analyzed by flow cytometry for apoptotic cells.

Results

Dose-dependent response of H9 cells to radiation. The dose-dependent response of H9 cells to radiation was determined by cell-survival assay. H9 lymphoma cells growing in cell suspension in RPMI-1640 medium were exposed to a single dose of 5 or 10 Gy and cell numbers were counted every other
day for 20 days with periodic medium change. On day 6, the untreated H9 lymphoma cells in RPMI-1640 medium showed an 88.5-fold increase in cell number, whereas only a 3.4-fold increase was observed after 5 Gy and a decrease (0.8-fold) in cell number was observed after 10 Gy (N=3). The growth rate of cells treated with 5 Gy was restored and became similar to the growth rate of the control group. In contrast, cells treated with 10 Gy did not recover and failed to proliferate within the complete observation period of 20 days.

Radioprotective effect of astrocytes and ACM. To determine whether the presence of astrocytes protect H9 cells from radiation-induced cell killing, limiting-dilution survival assay was performed. H9 cells growing on an astrocyte monolayer showed relative radioresistance compared with H9 cells alone (Figure 1). For example, the surviving fraction for H9 cells alone after 5 Gy was 3.0±0.2% (all data are reported as mean±standard error of the mean (SEM). In the presence of astrocytes, the survival fraction after 5 Gy was significantly enhanced to 16.8±3.7%. The p-value calculated by Student’s t-test was 0.02. To determine whether the protective effect of astrocytes requires cell-to-cell contact or results from the release of a soluble factor, media conditioned for three days on an astrocyte monolayer were added to H9 cells 48 h before 5 Gy of irradiation. H9 cells growing in ACM showed relative radioresistance compared with H9 cells growing in unconditioned RPMI-1640 medium. The surviving fraction after 5 Gy in unconditioned RPMI-1640 medium was 2.8±0.1%, in comparison to 8.7±0.1% with p=0.0002 (Figure 2) in ACM.

Intracellular GSH level. Radiation toxicity is mediated by reactive oxygen species (ROS) (22) and intracellular GSH is considered to be one of the front-line intracellular radical scavengers (23). The latter notion is supported by the observation that pre- and/or post-treatment GSH levels correlate with the sensitivity to different chemo-therapeutic agents and radiation (24-26). Therefore, it was hypothesized that GSH may play a role in astrocyte-mediated radioresistance of lymphoma cells. H9 cells were incubated for 48 h in either unconditioned RPMI-1640 or in ACM. Thereafter the cells were irradiated with 5 Gy and intracellular GSH levels were determined at 4 h after irradiation. Whereas a slight decrease in GSH level (not significant) was noted in H9 cells growing in RPMI-1640 medium at 4 h, a 1.15-fold increase (p=0.09, unpaired t-test) in GSH levels was noted when the cells were cultured in ACM (Figure 3).

Induction of apoptosis. H9 cells exhibited a basal level of apoptosis (14% of cells were apoptotic) and 4 h after irradiation (10 Gy) apoptosis increased to approximately 30% in unconditioned RPMI medium. However, when cells were grown in ACM, irradiation failed to induce apoptosis,
suggesting that ACM blocked the radiation-induced apoptosis in H9 cells (Figure 4).

**Cell-cycle distribution.** Irradiation of proliferating tumor cells may result in G2/M phase arrest of the cell cycle as a stress response (27). Flow cytometric analysis of H9 cells 48 h after 5 Gy of irradiation displayed an increase in the proportion of cells in G2/M phase when grown in ACM (Figure 5), as compared to cells growing in unconditioned RPMI medium. The proportion of H9 cells in G2/M phase increased from 15.5±0.7% to 34.7±1.4% after radiation in unconditioned RPMI-1640 medium and from 14.4±1.5% to 47.4±1.5% after radiation in ACM (p=0.03, unpaired t-test).

**Discussion**

PCNSL is a radioresistant type of non-Hodgkin lymphoma and treatment is often associated with neurologic toxicity. Improvement of local tumor control and reduction of treatment-induced toxicity are, therefore, major objectives in improving the outcome of this disease. Brain tissue consists of neurons and glial cells such as astrocytes. Several studies have shown that astrocytes can protect neurons from injury through a number of different mechanisms, such as their ability to uptake extracellular glutamate (28) or the catalase activity present within themselves (29). Noel and Tofilon (18) reported that astrocytes have the ability to reduce radiation-induced neuronal toxicity in vitro. The aim of the present study was to examine whether astrocytes can also protect lymphoma cells from radiation damage, thereby potentially contributing to the radioresistance of PCNSL. As a model system, the study used human astrocytes and the human H9 lymphoma cell line derived from T-cell non-Hodgkin lymphoma. The data presented herein indicate that astrocytes decrease the radiosensitivity of lymphoma cells in vitro.

It has been suggested that the protection of neurons from radiation is most likely mediated by an astrocyte-secreted protein of molecular weight >10kDa (18). In the study presented here, the presence of astrocytes and/or of ACM significantly protected H9 lymphoma cells from radiation-induced damage. Thus, in the clinical setting, the presence of astrocytes or astrocyte-related factors may be responsible for or contribute to the observed radioresistance of PCNSL.

Gamma irradiation causes oxidative stress characterized by an overproduction of ROS (22). These ROS interact with biological macromolecules and result in disturbance of biological structures and functions (30, 31). GSH is notable among the molecules known to take part in the defense of the organism against these ROS. GSH is a thiol tripeptide known to trap and fix ROS (32). Some studies mentioned a correlation between the level of the intracellular GSH after irradiation and the response to radiation (26, 33). Recently, Brouazin-Jousseaume et al. (26) confirmed that the evolution of intracellular GSH after irradiation is an important factor in determining the radioresistance of cells. In the present

![Figure 3. Intracellular total GSH levels in H9 cells growing in RPMI-1640 medium and in ACM after irradiation: H9 cells were maintained in either unconditioned RPMI-1640 medium or in ACM for 48 h and then irradiated with 5 Gy. Intracellular GSH levels were determined at 4 h after irradiation. A small increase in intracellular GSH levels was noted after irradiation in H9 cells growing in ACM (p=0.09, unpaired t-test). Data are expressed as the mean±SEM from three independent experiments.](image1)

![Figure 4. Induction of apoptosis in unconditioned RPMI-1640 medium or in ACM, after irradiation. H9 cells were maintained for 48 h in RPMI 1640 (black bars) or in ACM (grey bars) and then irradiated with a dose of 2, 5 or 10 Gy. Four hours later cells were collected and processed for apoptosis analysis. Data are expressed as the mean±SEM from three independent experiments.](image2)
study, the level of intracellular GSH of H9 cells incubated with ACM slightly increased at 4 h after 5 Gy of radiation. This may contribute to the mechanisms by which ACM confers radioresistance to H9 cells. Radiation exposure results in G1/S and mostly G2/M cell-cycle arrest. DNA-damage-sensing and -repair mechanisms operate in G2 to ensure DNA and chromosome integrity before mitosis and cell division (27). It is notable that no significant differences in cell-cycle distribution were observed in H9 cells growing in unconditioned RPMI-1640 medium versus cells growing in ACM. After irradiation, however, the proportion of H9 cells growing in ACM after irradiation. H9 cells were maintained for 48 h in either unconditioned RPMI-1640 or ACM and then irradiated with a dose of 5 Gy. Four hours after irradiation, the cells were washed and processed for flow-cytometry analysis for cell-cycle redistribution using propidium iodide staining. *The proportion of cells in G2/M phase at 4 h after irradiation differed significantly between cells growing in unconditioned or conditioned medium (p=0.03, unpaired t-test). The data shown are representative of three independent experiments.

In conclusion, the data presented in this report indicate that the radiosensitivity of lymphoma cells can be significantly reduced in the presence of astrocytes or ACM. These results suggest that the presence of astrocytes may decrease the susceptibility of PCNSL to injury induced by radiation and, thus, may contribute to the radioresistance of this disease observed in the clinic.


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