

Interaction of Radiation and Pemetrexed on a Human Malignant Mesothelioma Cell Line *In Vitro*

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Abstract. *Purpose:* To investigate the interaction of the cytotoxic activity of the combination of pemetrexed and radiation in a human mesothelioma cell line. *Materials and Methods:* The toxic effects of treatment with radiation and/or pemetrexed (PEM) on NCI-H28 cells were assessed by clonogenic assay. TUNEL/DAPI staining was used to measure the apoptosis index (AI). Thymidylate synthase (TS) protein expression was measured by Western blotting. *Results:* The D0 value of the combination treatment was lower than that for radiation, indicating its addition resulted in the synergistic effects with radiation. An increase in AI was observed with the combination compared with that observed after PEM alone. Radiation induced a time-dependent decrease in TS expression. Increased TS expression was observed after PEM, but the combination treatment decreased TS expression. *Conclusion:* Treatment with pemetrexed in combination with radiation has a supra-additive effect, partially due to the synergistic induction of apoptosis, which may be due to TS suppression, by the irradiation enhancing the effect of pemetrexed.

Malignant pleural mesothelioma (MPM) is a very aggressive malignancy that is radiation resistant. Even though MPM is treated with aggressive multimodality therapy, combining extrapleural pneumonectomy with chemotherapy and radiation therapy (1, 2) resulting median survival rates are 19 to 29 months. The crucial risk factor is chronic asbestos exposure and a long latency period between asbestos exposure and the development of MPM is characteristic. In

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Japan, the incidence of MPM is increasing because asbestos was used until the 1970s.

Pemetrexed (PEM) is a new pyrrolopyrimidine-based folate antimetabolite that is used for the clinical treatment of MPM (3). Unlike classic antifolates, PEM inhibits various folate pathway enzymes, including thymidylate synthase (TS), dihydrofolate reductase and glycinamide ribonucleotide formyltransferase. However, the primary target of its action is TS (4, 5). TS inhibition results in a decrease in the production of thymidine, which is necessary for DNA synthesis. Insufficient inhibition of TS may be the major mechanism of resistance to TS inhibitors in both preclinical models and the clinical setting. The measurement of TS expression in clinical tumor samples is used to predict overall clinical outcomes and the response to PEM (6). However, the radiobiological significance and the mechanism of the effects of chemoradiotherapy with PEM are not fully understood.

The aim of this study was to investigate the sensitizing effect of PEM in combination with irradiation using a human mesothelioma cell line, NCI-H28 (7). In addition, the mechanism responsible for the effects of the interaction of PEM and radiation was investigated from the viewpoint of TS expression.

Materials and Methods

Cell line and culture. The cell line used was derived from a human malignant pleural mesothelioma (NCI-H28 cells) and was obtained from the American Type Tissue Culture Collection (Manassas, VA, USA). The NCI-H28 cells were maintained in RPMI-1640 (Invitrogen Corporation, Carlsbad, CA, USA) medium supplemented with 10% heat-inactivated fetal calf serum (BioWest, Nuaille, France). For experimental use, 5ml of complete medium containing 500-10000 of exponentially growing NCI-H28 cells were seeded into 60 mm diameter Petri dishes at 37°C in a humidified atmosphere of 5% CO₂ and 95% air.

Radiosensitivity. Irradiation was started 12 h after incubation at 37°C to ensure sufficient time for any cell damage induced by the trypsinization procedure to disappear. Radiation was performed

using an X-ray machine (RX-650, Faxitron X-ray Corporation, Lincolnshire, IL, USA) operated at 130 kV and 5 mA with a 0.5 mm Al filter. The dose rate, which was quantified by thermoluminescent dosimetry at the irradiation distance (30 cm), was 87 cGy/min. Graded doses of radiation ranging from 1 to 7 Gy were used. The cells were irradiated in air at room temperature. The effect of irradiation on the cells NCI-H28 was assessed by clonogenic survival.

Pemetrexed sensitivity. PEM was provided by Eli Lilly and Company (Indianapolis, IN, USA), at a concentration of 25 mg/ml. The drug was then divided into aliquots and stored at -80°C and was freshly diluted in culture medium before each experiment. The experimental cultures were exposed to PEM for 2 h at 37°C using graded doses of PEM ranging from 50 to 250 ng/ml. Then, the PEM containing medium was removed and new pre-warmed complete RPMI-1640 medium containing serum was added to the dishes after the dishes had been washed with PBS. The effect of PEM treatment was assessed by clonogenic assay.

Effect of pemetrexed on radiation survival responses. The concentration of PEM required to reduce colony formation by 50% after 2 h treatment (the IC_{50} of PEM) was used to evaluate the sensitizing effect of PEM in combination with irradiation. The IC_{50} of PEM was 100 ng/ml (see Results). The experiments were started 12 h after the cell inoculation using the procedures described above. The NCI-H28 cells were irradiated with graded doses of radiation ranging from 1 to 7 Gy. After the irradiation, the NCI-H28 cells were immediately exposed to PEM for 2 h. After the PEM treatment, the PEM containing medium was changed under the conditions described above and the clonogenic assay was performed.

Clonogenic survival. After the treatment, the dishes were incubated in a CO_2 incubator at 37°C for 14 days to allow colony growth. The colonies were then fixed and stained with crystal violet solution and those with 50 or more cells were counted. The survival response/fraction was calculated as the colony survival after a given treatment relative to the mean value for the control Petri dishes. The plating efficiency of the cells was also determined for the non-irradiated cells. The D_0 definition was calculated from the reciprocal of the slope of the radiation dose response curve. All the experiments were performed in triplicate.

Terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) staining. The NCI-H28 cells were treated with 3 Gy irradiation alone, PEM alone (IC_{50} dose), or a combination of irradiation and PEM. Each treatment was performed according to the method described above. The TUNEL assay was performed using an ApopTag Fluorescein *In Situ* Apoptosis Detection Kit (Chemicon International, Temecula, CA, USA), as described previously (8). The cells were fixed with 4% paraformaldehyde in phosphate buffer (pH 7.4) 6, 12 or 24 h after each treatment. The fixed cells were then permeabilized with ethanol: acetic acid in a 2:1 ratio for 15 min at -20°C , before being washed twice in PBS for 5 min and incubated with ApopTag equilibration buffer for 5 minutes prior to terminal deoxynucleotidyl transferase enzyme linkage of dUTP-digoxigenin to the 3'-OH end of their DNA at 37°C for 60 min. The reaction was then terminated in stop/wash buffer at 37°C for 30 min. After washing the cells, the slides were treated with anti-digoxigenin-peroxidase for 30 min and mounted with Vectashield and 4', 6-diamidino-2-phenylindole (DAPI) (Vector

Laboratories, Burlingame, CA, USA). All the samples were assessed without any previous knowledge of the experimental details. All fluorescent cell images were obtained using a Keyence BZ-9000 microscope (Keyence, Osaka, Japan) equipped with a $20\times$ objective lens and a cooled Charge Coupled Device camera. The cells in four randomly chosen microscopic fields of each slide were examined. TUNEL-positive cells were considered to be undergoing apoptosis. The percentage of apoptotic cells (apoptotic index: AI) was determined by dividing the number of TUNEL-positive cells by the total number of DAPI stained cells. All the fluorescent images were analyzed with the BZ-II analysis application (Keyence). Both the TUNEL-positive cells and DAPI-positive cells were counted automatically using the BZ-II analysis application after modifying the application by haze reduction. All the data were reviewed by human observation. All the experiments were performed in triplicate.

Analysis of TS protein expression. The NCI-H28 cells were treated with 3 Gy irradiation alone, PEM alone (IC_{50} dose), or a combination of irradiation and PEM. Each treatment was performed as described above. From 1-24 h after each treatment, the total cellular protein was isolated from the cells in lysis buffer containing proteinase inhibitors and equal quantities (50g) of protein were separated by SDS-PAGE (8-16% Tris/glycine gels purchased from Novex, Frankfurt, Germany) and electroblotted onto a nitrocellulose membrane. Immunoblotting was performed using the primary antibodies, anti-human TS (Abcam, Cambridge, UK) and anti-beta actin (Santa Cruz Biotechnology, SA, USA), followed by appropriate horseradish peroxidase-conjugated secondary antibodies (Amersham Life Sciences, Bucks, UK). The proteins were detected using enhanced chemiluminescence according to the manufacturer's instructions (Amersham Life Sciences).

Statistical analyses. Multiple-comparisons analysis was performed by one-way analysis of variance, using the Tukey-Kramer method. All the analyses were performed using SPSS software (version 11.0, SPSS Institute, IL, USA). The level of significance was set at $p=0.01$.

Results

Radiosensitivity. Data on the effects of radiation are shown in Figure 1. The D_0 value for radiation sensitivity, which was calculated by taking the reciprocal of the slope of the radiation dose response curve, was 1.76 ± 0.28 Gy.

Pemetrexed sensitivity. The survival response of the NCI-H28 cells to 2 h exposure to graded doses of PEM is shown in Figure 2. The IC_{50} of PEM was 100 ng/ml, which was used as the dosage for the PEM and radiation combination treatment.

Effect of pemetrexed on radiation survival responses. Figure 3 shows the survival curves for the NCI-H28 cells treated with radiation alone or PEM treatment (IC_{50} dose) combined with a radiation dose ranging from 1 to 7 Gy. The D_0 value for this combination was 0.97 ± 0.06 Gy and was significantly lower than that for radiation alone ($p<0.01$).

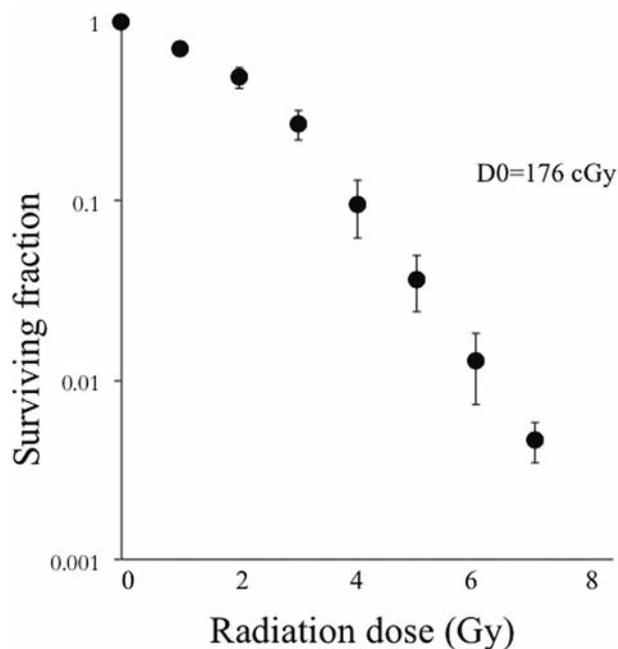


Figure 1. Clonogenic survival of NCI-H28 cells after irradiation. Data are presented as the mean \pm SD.

Apoptosis evaluation after radiation with/without pemetrexed.

The AI of the untreated cells was 0.0%. No differences in the AI were found 6 h after each treatment (data not shown). Figure 4 shows positive staining of DAPI and TUNEL at 12 h after. As figure 5, the AI at 12 and 24 h after irradiation were 0.2% and 2.2%, respectively. The AI was not significantly increased by irradiation alone. The AI at 12 and 24 h after PEM exposure were 20.2% and 16.7%, respectively. The AI for PEM exposure was significantly increased compared with that of the untreated control ($p < 0.01$). The AI at 12 and 24 h after treatment with PEM combined with irradiation was 30.6% and 95.8%, respectively. The AI for PEM combined with radiation was significantly increased compared with that for the untreated control ($p < 0.01$). The AI at 12 h after treatment with PEM combined with irradiation was not significantly increased compared with that for PEM exposure alone; however, 24 h after treatment with PEM combined with irradiation, the AI was significantly increased compared with that for PEM exposure alone ($p < 0.01$).

Analysis of TS protein expression after treatment with radiation with/without pemetrexed. In time-course analysis, the TS protein expression of the NCI-H28 cells was decreased after treatment with 3 Gy irradiation alone. Figure 6 demonstrates representative immunoblot patterns of the TS protein expression at 1 to 24 h after the irradiation. After PEM exposure, the TS protein expression increased (Figure 7) while after treatment with a combination of irradiation and

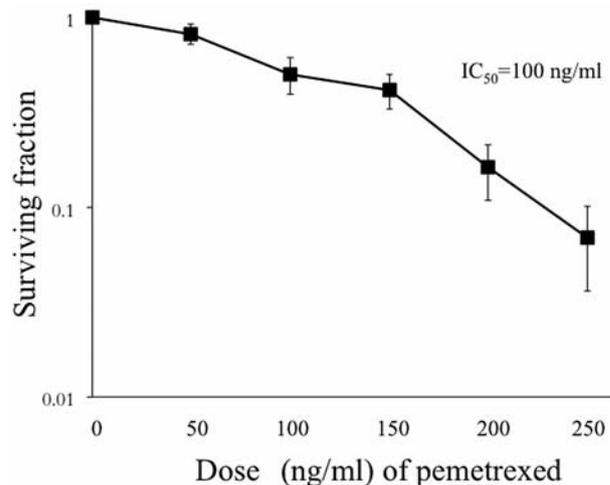


Figure 2. Clonogenic survival of NCI-H28 cells after pemetrexed exposure. Data are presented as the mean \pm SD.

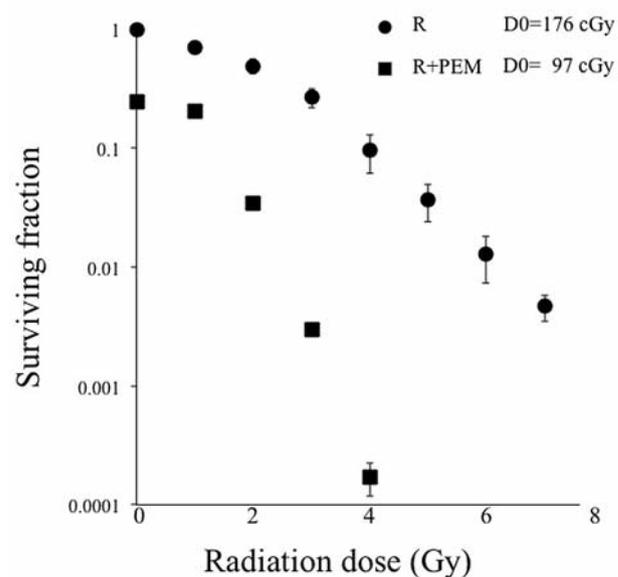


Figure 3. Clonogenic survival of NCI-H28 cells after irradiation alone or in combination with pemetrexed. Data are presented as the mean \pm SD.

PEM the TS protein expression increased to a lesser extent compared to the control. All the experiments were performed in triplicate, and consistent results were obtained.

Discussion

Häkkinen *et al.* examined the radiosensitivity of six mesothelioma cell lines (9) and found that the mean surviving fraction at 2 Gy (SF_2) of the six cell lines was 0.6 ± 0.24 , indicating that they were more radioresistant than fibroblasts (SF_2 of 0.49 ± 0.04). The present results showing

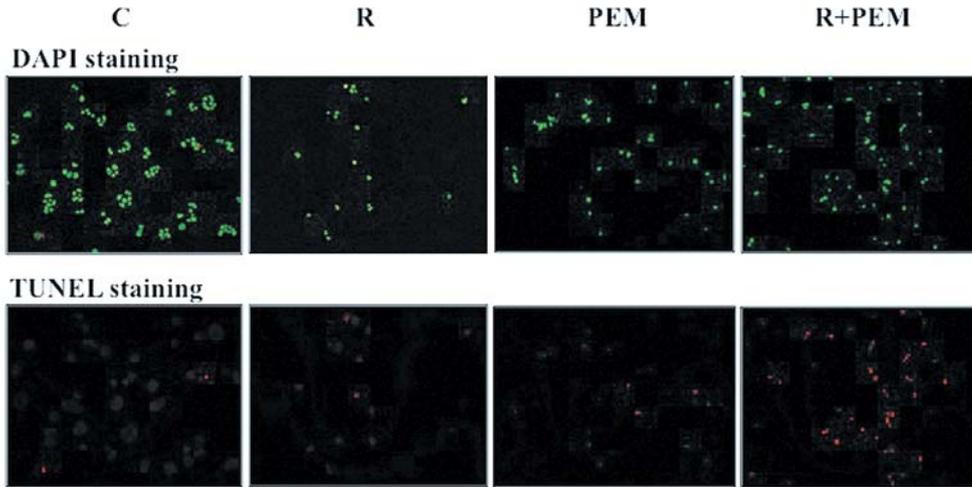


Figure 4. DAPI and TUNEL staining images of the same field after irradiation alone or in combination with pemetrexed (C: Control, R: radiation, PEM: pemetrexed).

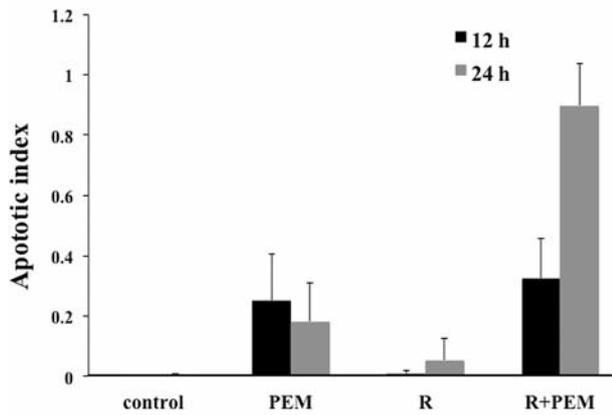


Figure 5. Apoptotic NCI-H28 cell assays performed at 12 h and 24 h after treatment with irradiation or irradiation combined with pemetrexed (C: Control, R: radiation, PEM: pemetrexed).

that the NCI-H28 cells displayed a D0 value of 1.76 ± 0.28 and an SF₂ value of 0.49 ± 0.06 indicated that the NCI-H28 cells were quite highly radiosensitive for a mesothelioma cell line and showed similar radiosensitivity to M14 cells, which had the highest radiosensitivity among the mesothelioma cell lines examined in Häkkinen's study.

In the present study, PEM toxicity was clearly observed in the NCI-H28 cells exposed for 2 h at concentrations >150 ng/ml. As indicated in Figure 3, the significantly smaller D0 value for the combination of PEM and radiation compared with that for radiation therapy alone indicated that the combination of PEM and radiation had synergistic effects compared with the additive effects of PEM alone plus radiation alone. Hence, this combination modality may be useful for clinical application to the treatment of MPM.

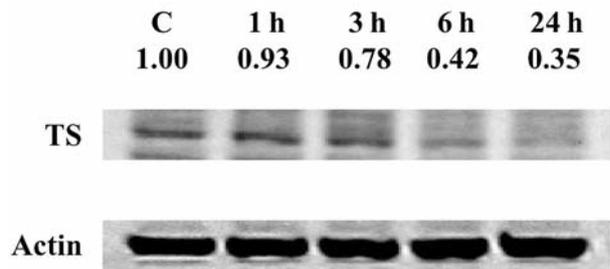


Figure 6. Time-course analysis of TS and actin expression after 3 Gy irradiation. The relative intensities of the TS bands are indicated at the bottom of the lanes.

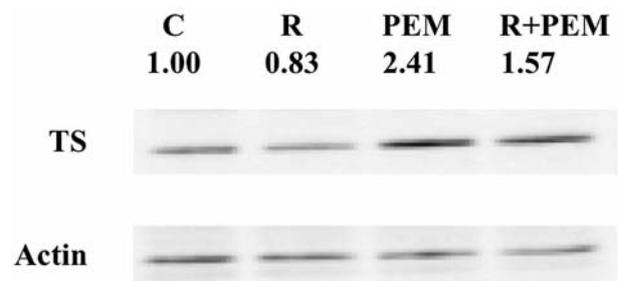


Figure 7. Changes in TS and actin expression after irradiation and/or pemetrexed exposure. The relative intensities of the TS bands are indicated at the bottom of the lanes (C: Control, R: radiation, PEM: pemetrexed).

Apoptosis plays an important role in cell death due to radiation as shown by Stephens *et al.* in two murine tumor models (10). In the present study, AI after irradiation was similar to the AI in the untreated control while the AI at 12 and 24 h after treatment with PEM exposure alone or PEM

combined with radiation were significantly increased compared with that of the untreated control. In particular, the AI at 24 h after treatment with PEM combined with irradiation was significantly increased compared with that for PEM exposure alone, suggesting that apoptosis played an important role in the cell death induced in the NCI-H28 cells treated with radiation and PEM.

After irradiation, TS expression decreased over time. To the best of our knowledge, no previous studies have reported the effect of irradiation on TS expression. TS expression was up-regulated after PEM exposure, as was reported previously (11, 12). TS up-regulation was recognized after treatment with PEM combined with irradiation, however, the extent of this up-regulation was smaller than that produced by PEM alone. Hwang *et al.* (13) demonstrated TS suppression after treatment with a combination of fluorine pyrimidine and irradiation induced an imbalance in the nucleotide pool which resulted in the inhibition of DNA synthesis/repair and the generation of DNA fragmentation and enhanced cell death. Thus, TS suppression by irradiation seems to induce apoptosis and may be responsible for the synergistic effect observed after treatment with PEM combined with radiation.

The synergistic effect of treatment with radiation and chemotherapeutic agents, such as fluoropyrimidines or cisplatin is well known. Britten *et al.* evaluated the activity of PEM against a broad range of human tumors including renal cell carcinoma, hepatocellular carcinoma, mesothelioma and pancreatic carcinoma and found that PEM activity was not completely cross-resistant with those of cisplatin, fluorouracil, irinotecan and paclitaxel (14). Therefore, treatment with a combination of radiotherapy and PEM might not only be a first-line treatment for primary MPM, but could also be an effective second- or third-line treatment for recurrent malignancies after first-line chemotherapy.

In conclusion, treatment with a combination of PEM and radiation has a synergistic cytotoxic effect on human MPM cells and the down-regulation of TS expression by radiation may induce apoptosis which may be responsible for the observed synergistic effect.

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