Abstract. Background: Amifostine is an important broad spectrum cytoprotective agent approved for protection during fractionated radiotherapy. The daily dose of amifostine used, however, is arbitrarily chosen and low compared to the actual tolerable dose. Materials and Methods: Cohorts of mice (n=6) were treated with one up to 4 consecutive fractions of 6 Gy of whole-body γ-irradiation ($^{60}$Co), supported with increasing daily subcutaneous (s.c.) doses of amifostine (10 mg/g-300 mg/g). Survival and weight loss were monitored. Histopathological analysis was performed in mice receiving 3×6 Gy. Results: By increasing the amifostine dose from 13 to 50 mg and to 160 mg/g, the 50% lethal dose of radiotherapy increased from 2×6 Gy to 3×6 Gy and to 4×6 Gy, respectively. To keep the median weight loss to less than 25% of the initial weight, the dose of amifostine demanded was 23 mg/g, 68 mg/g and 121 mg/g, for 2×6 Gy, 3×6 Gy and 4×6 Gy, respectively. Histopathological analysis revealed a net protection of the liver and intestine of the mice receiving amifostine. Extensive and multiple vacuolar degeneration of the cytoplasm with focal necrosis of hepatocytes and loss of the intestinal villi was the most striking finding in the dying mice treated without amifostine. Conclusion: Taking into account the strong association of daily amifostine dose with cytoprotective efficacy and that a slight reduction of the daily amifostine dose can substantially reduce the clinical protective effect during fractionated radiotherapy, it is suggested that randomized trials should be re-appraised adopting amifostine schedules close to the maximum tolerable dose.

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In the current experimental study, hypofractionated whole-body radiotherapy (6 Gy) was used to assess the effect of amifostine in preventing body weight loss and death from increasing number of radiation fractions in mice.

Materials and Methods

Animal care and handling. Animal care and handling was carried out according to the guidelines set by Directive 86/609/EEC. All the experimental procedures were approved by the Veterinary Direction for Animal Research in the Department of Experimental Surgery at the Democritus University of Thrace. Male mice (Balb/c), 14 to 16 weeks of age (33±2 g), were kept under normal conditions concerning ambient temperature (21-23˚C), diet and tap water ad libitum and were maintained on a 12 h light:12 h dark cycle.

Experimental design. Groups of male mice were irradiated according to three different fractionated radiotherapy schedules: A: Two consecutive fractions of 6 Gy [control (n=6), 10 mg/kg of amifostine (n=6), 25 mg/kg (n=6), 50 mg/kg (n=6), 100 mg/kg (n=6) and 200 mg/kg (n=6)]. B: Three consecutive fractions of 6 Gy [control (n=6), 50 mg/kg of amifostine (n=6), 75 mg/kg (n=6), 100 mg/kg (n=6) and 200 mg/kg (n=6)]. C: Four fractions of 6 Gy [control (n=6), 200 mg/kg (n=6), 250 mg/kg (n=6) and 300 mg/kg (n=6)]. An additional group of 6 mice received 1 fraction of 6 Gy but as all the mice survived without amifostine, no further experiments were performed at this dose.

The mice were exposed to whole body γ-radiation at a dose per fraction of 6 Gy (Cobalt 60). The fractions were delivered on 4 consecutive days, 24 h apart. Amifostine (Ethyol®, Pinnacle Biologics, IL, USA) at a dose of 10-200 mg/kg was injected s.c. 30 min before irradiation. The volume of each injection dose was equal. The body weight of the mice was recorded daily for 30 days or till death.

Radiobiological considerations. Each fraction of 6 Gy is biologically equivalent to a normalized dose of 10 Gy given with 2 Gy fractions, for most normal tissues with an average of α/β=4 Gy. In this way, 4 consecutive fractions of 6 Gy correspond to 32-40 Gy. Assuming a λ-value of 0.2 Gy for normal tissues and an acceleration of 3 weeks, this gives an equivalent of 37-45 Gy of standard fractionated radiotherapy. Such a dose, given to the whole body, is very high as it is equivalent to the total dose delivered for

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Figure 1. Kaplan-Meier survival curves of mice treated with 2×6 Gy (a), 3×6 Gy (b) and 4×6 Gy (c) and sigmoid radiation dose-dependent survival according to the amifostine dose (d).
post operative intent in limited body regions (e.g. pelvis) and about 2/3 of a radical radiotherapy scheme delivered to smaller body areas. Details of the radiobiological calculation of the normalized total dose (NTD) have been previously reported (8,9).

Pathology study. Three additional groups of mice treated with the 3×6 Gy schedule supported with 200 mg/kg of amifostine (n=4) or without amifostine (n=4) and unirradiated mice (n=4), were sacrificed on day 7 (before the expected day of death of the mice not receiving amifostine). Their organs (lungs, liver, kidney, heart and colon) were collected, put in formalin 10% and sent for pathological examination. Microscopic examination was performed on hematoxylin-eosin tissue sections. In addition, liver tissues were stained for periodic acid Schiff reaction with and without diastase digestion (PAS and PAS-d, respectively).

Statistical analysis. Statistical analysis was performed using the GraphPad Prism 5.0 (GraphPad Software Inc., San Diego, CA, USA). Paired or unpaired two tailed t-test was used for testing relationships between continuous categorical variables, as appropriate. Kaplan-Meier curves were used to assess the survival of the mice. A p-value of <0.05 was regarded as significant.

Results

Survival analysis. Figure 1 shows the survival analysis according to the radiation and amifostine dose groups. One fraction of 6 Gy allowed 100% survival of mice, even when not supported with amifostine (data not shown). By adding one additional fraction, 25 mg/kg of amifostine were necessary to achieve 100% survival. To increase the number of 6 Gy fractions from 2 to 3, the dose of amifostine needed to be increased to 75 mg/kg to totally abrogate the lethal effect of radiotherapy. A dose of amifostine between 200-300 mg/kg provided an important protective effect against 4×6 Gy, as 11/18 mice survived, but no total abrogation of death was noted. Sigmoid dose-response plotting showed that by increasing the amifostine dose from 13 to 50 mg, the LD50 of radiotherapy increased from 2×6 Gy to 3×6 Gy (NTD 20 to 30 Gy, respectively). The dose of 200 mg/kg was able to protect more than 50% of the mice treated with 4×6 Gy (NTD 40 Gy), the data implying an LD50 of 160 mg/kg.
Weight loss analysis. Figure 2 shows the weight loss according to the radiation and amifostine dose groups. To avoid massive weight loss of mice receiving 2×6 Gy, a daily amifostine dose of 25 mg/kg was necessary. The weight loss increased significantly by decreasing the amifostine dose from 25 mg/kg to 10 mg/kg or 0 mg/kg \((p<0.0001)\). In the mice receiving 3×6 Gy, the amifostine dose required to avoid significant weight loss was 75 mg/kg/day. Lower doses were linked with increased weight loss \((p<0.0001)\). In mice receiving 4×6 Gy, a significant loss of weight was noted even at doses as high as 300 mg/kg/day, but there was a significant protection compared to controls \((p<0.002)\).

Sigmoid dose-response analysis showed that to keep the median loss of weight at less than 25% of the initial weight, the dose of amifostine demanded was 23 mg/kg, 68 mg/kg and 121 mg/kg, for 2×6 Gy, 3×6 Gy and 4×6 Gy, respectively.

Histological evaluation. Histological analysis was performed 7 days after irradiation and so reflected very early radiation tissue damage. There was a clear difference of tissue damage observed between the mice receiving radiotherapy with or without amifostine. The most striking effect was on the liver and intestine (Figure 3). Hepatocytes of the mice treated with amifostine showed the formation of multiple small discrete vacuoles in the cytoplasm \((PAS\) and \(PAS-d\) negative) with focal necrosis of liver cells. Similar changes were not observed in the normal hepatocytes. In the irradiated mice without amifostine, there was a striking fusion of the vacuoles, creating confluent spaces occupying the whole cytoplasmic area, indicating a total vacuolar degeneration of the liver.

Pathological alteration of the structure of the intestinal epithelium was seen only in the mice treated with radiation alone. Complete loss of the intestinal villi was evident in the colon of the mice treated with radiation alone, while these remained unchanged in mice receiving amifostine cytoprotection.

Renal cells irradiated without amifostine showed cytoplasmic degeneration, with crumpling of the luminal cell surface with portions of the cytoplasm entering the lumen of...
the urinary tubes. The lung tissue of the mice treated with amifostine showed no particular differences compared to the normal lung. In contrast, cytoplasmic swelling of the alveolar cells with thickening of the alveolar walls was evident in the mice irradiated without amifostine. No apparent histological changes were noted at the examination of the heart and brain of the mice.

Gastrointestinal radiation damage was, therefore, considered the most likely cause of the early death of the mice receiving 3×6 Gy without amifostine.

Discussion

Although a large number of experimental studies with cell lines and animals have been published examining the cytoprotective effect of amifostine, the dose of amifostine has rarely been tested against the protective effect. In 1982, Stewart and Rojas published an interesting study on skin radioprotection (11). By increasing its dose from 200 mg/m² to 400 mg/m², the amifostine protection factor increased from 1.07-1.28 to 1.20-1.62. Rubin et al. in endothelial cell line experiments observed that following 2 Gy of radiation, the colony formation of cells increased proportionally by a factor of about 2 and 3 when the cells were exposed to 1 nM and 4 nM of WR-1065 (active form of amifostine), respectively (12). More recently, Cassatt et al. showed that the radioprotective effect of amifostine against oral mucositis in rats was dose dependent, increasing sharply at 200 mg/kg compared to 50-100 mg/kg (13, 14). The protective effect was similar regardless of the route of administration (i.v. or s.c.).

In the current study, in order to increase the number of radiation fractions from 2 to 3 and to 4, a disproportionally higher amifostine dose was necessary in order to prevent body weight loss and death. The amifostine dose to allow survival of 50% of the mice, following a standard fractionation equivalent of 20 Gy, 30 Gy or 40 Gy of radiation was 13, 50 and 160 mg/kg/day respectively. A 2-fold and 7.5-fold higher daily dose was necessary to protect against 3 and 4 fractions, respectively. It seems that the accumulated damage induced by each fraction increases throughout the course of radiotherapy and this excess damage can be neutralized by increasing the daily amifostine dose. It is unknown, however, whether protection is achieved by reducing the overall damage induced by each fraction or by reducing an unrepairable component that persists until the subsequent fraction or both. Amifostine certainly has both scavenger and DNA-repair activity so both pathways are plausible (15, 16).

Looking at the survival curves, it was evident that slightly a lower amifostine dose per fraction failed to confer any protection against death. A dose difference of 15-25 mg/kg was enough to confer or to abolish the radioprotective effect. It is, therefore, postulated that during fractionated radiotherapy, an ‘appropriate’ amifostine dose per fraction is necessary, while slightly lower doses will result in no overall effect.

However, the maximum usable amifostine dose is defined by the maximum tolerable dose for the animal and, moreover, by the maximum dose that can be metabolized and used by its tissues. In mice, doses higher than 1,000 mg/kg have been administered, but death frequently occurred. The LD₅₀ for WR-2721 was estimated to be 1,025 mg/kg and the mice died within 4 days following i.p. injection (17). The death/dose curves were very steep, showing a low death rate for doses lower than 900 mg/kg. In humans, the amifostine dose resulting in dose-limiting toxicities has not been identified. In pharmacokinetic studies in experimental animals, the concentration of WR-2721 and its metabolites increased sharply after amifostine administration (18). In humans following a dose of 150 mg/m² i.v., 94% of WR-2721 disappears from the plasma within 6 min (19). Additional pharmacokinetic studies in humans have shown that by increasing the dose of amifostine, an increasing accumulation of the unmetabolized form is noted in the plasma, which is compatible with saturable kinetics of the hydrolysis of the drug (20).

The recommended dose of 910 mg/m² before cisplatin infusion resulted in a substantial rate of side-effects, including hypotension and vomiting (1). It seems that the dose of 910 mg/m² results in excess accumulation of the unmetabolized drug in the plasma of patients, which results in the excess side-effects without augmenting the intracellular content of the active metabolites. The dose of 910 mg/m² should rather be considered a dose close to the maximum tolerated and effective dose for humans. The repeated daily usage of this dose has not yet been tested. In our experience, 60% of patients can tolerate a flat dose of 1,000 mg well and tolerance is not dependent on the body surface or weight (8-10). This is certainly a higher dose than that used up to now in randomized clinical trials, which ranged from 300-500 mg.

Amifostine is a strong radioprotector of normal tissues, allowing survival of mice and tissue recovery after at least a doubling of the otherwise lethal total-body radiation dose. Taking into account the strong association of daily amifostine dose with the cytoprotective efficacy, and that even a slight reduction of the daily amifostine dose can reduce the clinical protective effect on fractionated radiotherapy, the amifostine dose must increase disproportionately to abrogate the damage induced by increasing number of fractions. The window for such protection to be substantiated is very narrow, implying that small variations of the amifostine dose may render radioprotection ineffective, stressing the importance of pursuing an optimal amifostine dosage for clinical use. It is suggested that randomized trials should be re-appraised adopting amifostine schedules close to the maximum tolerable dose.
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References


