Histologic morphometry confirms a prophylactic effect for hyperbaric oxygen in the prevention of delayed radiation enteropathy

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Feldmeier JJ, Davolt DA, Court WS, Onoda JM, Alecu R. Histologic morphometry confirms a prophylactic effect for hyperbaric oxygen in the prevention of delayed radiation enteropathy. Undersea Hyper Med 1998; 25(2):93–97.—In a previous publication (Feldmeier et al., Radiother Oncol 1995; 35:138–144) we reported our success in preventing delayed radiation enteropathy in a murine model by the application of hyperbaric oxygen (HBO_2). In this study we introduce a histologic morphometric technique for assessing fibrosis in the submucosa of these same animal specimens and relate this assay to the previous results. The histologic morphometry, like the previous gross morphometry and compliance assays, demonstrates a significant protective effect for HBO_2. The present assay is related to the previous assays in a statistically significant fashion. The predictive value for the histologic morphometric assay demonstrates a sensitivity of 75% and a specificity of 62.5%. The applicability of this assay to other organ systems and its potential superiority to the compliance assay are discussed.

hyperbaric oxygen, radiation injury, histologic morphometry, enteropathy, radioprotectors

Radiation therapy is commonly used with curative intent for pelvic tumors either as an adjunct to surgery or as a primary modality (1–4). Radiation therapy is also used for the treatment of abdominal tumors but its application in this site is limited to the inherently poor tolerance of abdominal organs to high doses of radiation (5,6). Because of its susceptibility to radiation injury, the small bowel is one of several organs that limit our ability to deliver potentially curative doses of irradiation in abdominal and even occasionally in pelvic tumors (7). Some radiation effects on the small bowel are seen acutely during and just after irradiation. Fortunately, these are usually self-limited and are treated symptomatically (8). Delayed radiation injury of the small bowel is uncommon with present clinical practices, but when it occurs it is generally not self-limited, and in its most severe form may be life-threatening and require surgical intervention of resection or bypass of the affected segment (9,10). A consistent finding in delayed radiation damage is an increase of stromal fibrosis of the affected organ (11). In the small bowel, this increase in fibrosis is seen primarily in the submucosa (12). In a previous publication (13) we reported successful intervention with hyperbaric oxygen (HBO_2) in preventing delayed radiation enteropathy in a murine model. In this earlier report we identified the presence and graded the severity of enteropathy by applying a scoring scheme for gross anatomical manifestations of enteropathy and by conducting an analysis of bowel compliance using a specially constructed stretch apparatus. In our present study we apply a histologic morphometric analysis to segments of ileum from the same animals and relate these findings to the previous assay systems used to quantify the presence of enteropathy. We endeavor to quantify the amount of fibrosis in the submucosa of the animals previously studied.

MATERIALS AND METHODS

As reported in our previous publication (13), 50 young adult female C3W/HEN mice received identical whole abdominal and pelvic irradiation consisting of 3,000 cGy in 10 fractions over 2 wk. Radiation was delivered using a 250 kVp orthovoltage beam through a single anterior portal. Animals were anesthetized and irradiated five at one time in a jig mounted to the machine head. In vivo exposure measurements were made using a Victoreen R-meter which verified that the exit dose matched the planned value. One half of these animals (group II) also completed a course of HBO_2 beginning 7 wk after the radiation exposure. HBO_2 consisted of 30 daily treatments over 6 wk. Group II animals were exposed to HBO_2 in a small steel hyperbaric chamber (manufacturer Bethlehem Steel) graciously loaned by the United States Air Force. The chamber doors were sealed and 100% medical grade oxygen was supplied from

a bottled, pressurized source through a pressure regulator until the ambient pressure reached 2.4 atm abs . A continuous flow of O_2 was maintained while the environment of the chamber was pressurized to prevent the accumulation of CO_2. Animals breathed the O_2 in the chamber at an ambient pressure of 1,824 mmHg for 90 min. The O_2 was then allowed to slowly exhaust under its own force until equilibrium with the outside environment was achieved. Animals were then removed from the chamber and returned to the animal colony for maintenance. Each HBO_2 exposure lasted 100–110 min, including the exposure at pressure as well as the compression and decompression phases. Group I animals (radiation only) as well as group II animals (radiation plus HBO_2) were housed in the animal colony along with three additional animals, group III who received neither HBO_2 nor radiation. This small control group served to establish reference values for all of our analysis parameters. Animals had access to water and standard laboratory mouse chow ad libitum in the animal colony.

Animals were monitored and weighed on a weekly basis to detect any differences in small bowel digestive and absorptive function among the three groups. After 7 mo, all remaining animals were euthanized.

The previous analysis included gross inspection of the abdominal–pelvic cavities. The compliance analysis measured the potential resting circumference of the ileum (C_o) and the force per unit stretch of the mouse ileum. Before the measurement run, a conditioning regimen was done which fatigued muscular activity and isolated the influence of the elastic constituents (principally collagen) of the intestinal loop. Analysis of these assays demonstrated that group II (HBO_2-treated animals) had statistically different values for each assay compared to group I, and each of these showed a protective effect by HBO_2 for all measures of incidence and severity of enteropathy. A segment of ileum just proximal to those used for the stretch analysis was also taken from each animal, preserved in formalin, embedded in paraffin, and prepared as a histologic specimen on a glass microscope slide. These slides were stained with Masson's trichrome stain which selectively stains collagenous components blue and non-collagenous components red. Images of these slides were digitally captured using a low power (40×) magnification system attached to a video system using the Snappy video capture system. The morphometric analysis was blinded in the following fashion: Digitized histologic preparations were randomly assigned a sequential identification number which was referenced against the master listing of individual animals and group membership. This list was not available to either individual independently accomplishing the morphometry.

Once images were digitized, morphometry was accomplished using a personal computer and a commercially available image analysis software package (Mocha by Jandel Scientific). In general, the entire cross section of an individual bowel segment was analyzed. To account for instances in which the bowel was cut on a diagonal or when the entire segment was not cut in cross section, the relative area of blue (collagenous) to red (non-collagenous) stained components were analyzed. For consistency and because fibrosis within individual villi was subject to artifact (loss during microtoming), the analysis was restricted to the annular band of fibrosis in the submucosa. In this fashion, results were normalized and were believed to be more valid than absolute areas that would depend on the cut and orientation of the slide.

If area determinations varied by more than 10% between the two independent investigators, the difference was resolved by conference. When determinations differed by less than 10%, the higher value for relative areas was used in the analysis. The morphometric analysis process is shown in Figs. 1–3.

RESULTS

Forty-four histologic specimens were available for analysis (21 from group I and 23 from group II). As with the previous assays (gross morphometry, potential resting circumference, and compliance) the histologic morphometric analysis showed a statistically significant difference between group I (radiation only) and group II (radiation plus HBO_2) animals. For our group of unirradiated controls, the average percentage stained positive for collagen was 7.5% (SEM = 1.5%). For group I, this percentage was 9.9% (SEM = 0.752%), and for group II the percentage was 7.3% (SEM = 0.537%). Group II animals had a statistically significant decrease in relative areas of fibrosis as compared to group I animals ($P = 0.0057$, t test). Figure 4 demonstrates the relative frequencies of ranges of area stained positive for both groups I and II. Within each group there was a wide variation as illustrated in Fig. 4. This depiction suggests a shift to the left for group II values as compared to group I. On further examination, we find that 70% of group I animals have values greater than or equal to 8.2%, whereas only 35% of group II animals have like values.

To evaluate the predictive value for enteropathy of these area determinations, a chi-square analysis was also accomplished with a 2×2 table as depicted in Table 1. In this table an "observed incidence" for enteropathy was considered to have occurred if any of the previous assays (gross morphometry or either stretch parameter) were consistent with enteropathy. For the purpose of this analysis we excluded the finding of fragile adhesions if these had been

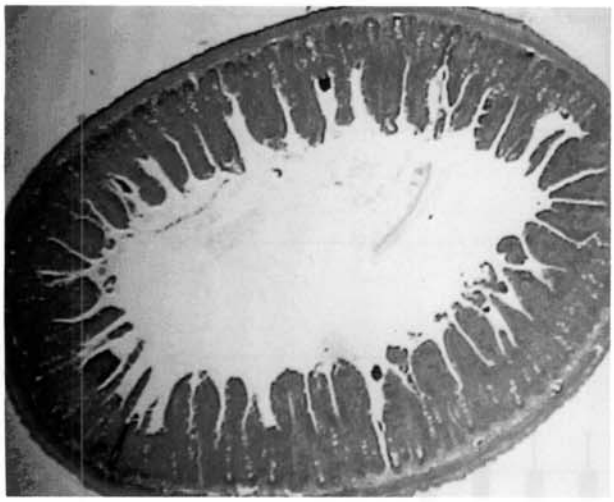

FIG. 1—A cross section of mouse ileum (40× magnification) stained with Masson's trichrome stain.

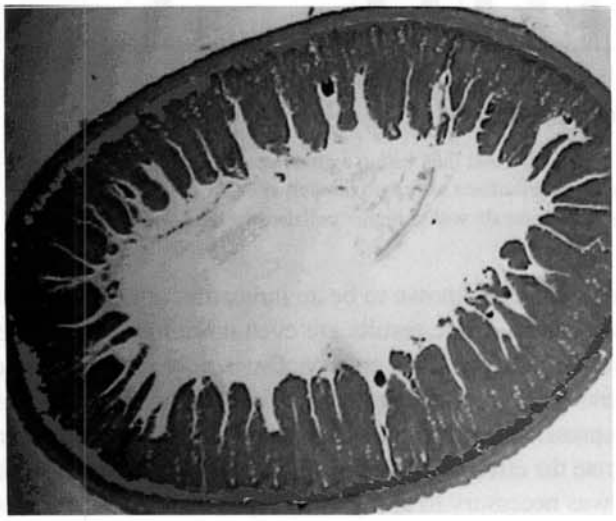

FIG. 2—The overlay applied to the annular rim of fibrosis within the submucosa, which defines that portion of cross-sectional area positive for collagen and on which the area determination is made.

the only gross morphometric finding for an individual animal. For the histologic morphometry, a positive prediction for enteropathy was considered to be any percentage of positive collagen stain that equaled or exceeded 8.2%. The *P* value for this chi-square analysis equals 0.0290, or in other words there is a 97.1% probability that the histologic morphometry is related to the other assays.

Using the same values in Table 1, we can determine the specificity and sensitivity of the histologic morphometric analysis. The sensitivity of the test, that is the likelihood that morphometry predicts enteropathy when the other analyses indicate its presence, is 15/20 or 75%. The specificity of the test, that is the likelihood that morphometry is correct when it predicts enteropathy, is 15/24 or 62.5%.

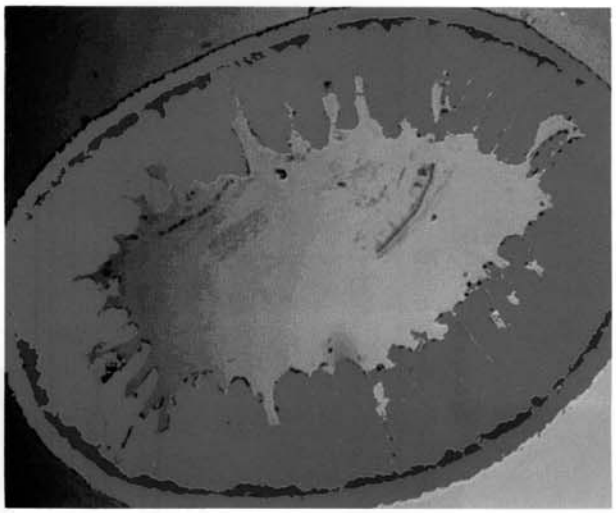

FIG. 3—The two overlays in place: one defines the collagenous components (as in Fig. 2), the other defines the non-collagenous components for relative area determinations.

The results of the morphometry assay were also compared by linear regression to the previous numeric values of C_o (resting circumference) and force per unit stretch. The linear regression for C_o against the percentage stained positive for collagen was statistically significant at a *P* value of 0.0020. The linear regression of morphometry with force determined by the previous stretch assay did not achieve statistical significance but showed a trend toward a relationship with the *P* value equal to 0.1181.

DISCUSSION

Morphometry utilizing Masson's trichrome stain has been previously reported as a measure of interstitial fibrosis in the setting of renal transplantation and the use of immunosuppressive therapy (14) or following percutaneous nephrostomy dilation (15). Histologic morphometry has also been used as a method of quantifying changes in the aged heart (16), in the myocardium and the coronary vessels of animals with renovascular hypertension (17), and to quantify damage to the pancreas in the setting of chronic pancreatitis (18). Langberg and associates (19) have previously reported a study wherein a semiquantitative evaluation of fibrosis within the submucosa of rat intestine was used as a deteminant for late radiation damage. To our knowledge, our study is the first report using Masson's trichrome stain and the semi-automated area determinations available to PC users with commercially available image analysis software in the assessment of fibrosis of the small bowel as a result of fractionated radiation exposure.

The results of this histologic morphometric analysis are

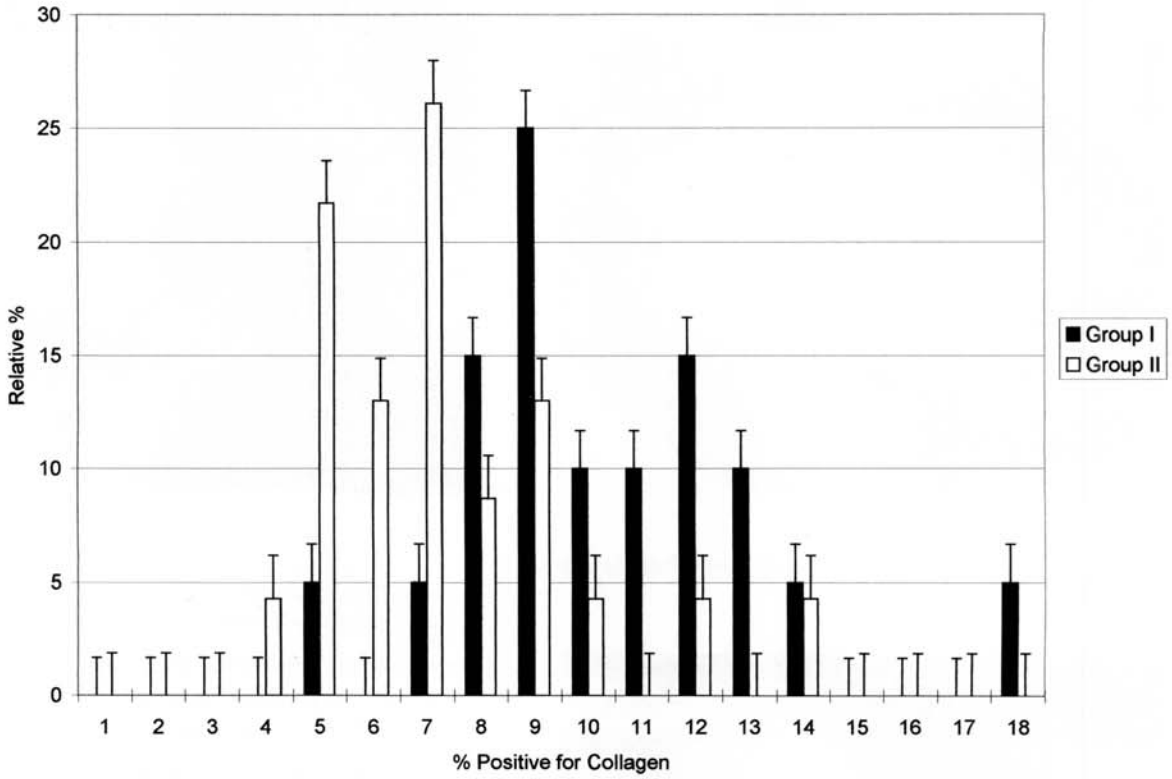

Fig. 4—The relative percentage of animal specimens within each group that falls within a given percentage range of relative areas stained positive for collagen. For each group the distributions are approximately normal. Group I plots (*open columns*) are shifted to the right, demonstrating more animals with a higher collagen content. *Solid columns* represent group II.

in good agreement with our previously reported results. Once again, this analysis suggests that HBO_2 was highly successful in preventing enteropathy or at least the deposition of increased collagen within the small bowel submucosa, which is known to be an indication of delayed radiation effect. These results are even more impressive when we recall that delayed enteropathy is not expressed homogeneously along the length of the bowel and is patchy in its expression, with frequent skip areas that do not demonstrate the effects of radiation injury (13,20). In our methods it was necessary to apply the morphometric analysis to an adjoining segment of ileum and not to the exact same segment, since the stretch analysis distorts the histologic morphometry of that segment. We also found the morphometric analysis to be less tedious and time consuming compared to the stretch analysis. An attractive feature of the stretch analysis as developed by Peck and Gibbs (21) is that it directly measures the lumen caliber and compliance of the bowel segment studied. These two attributes are very important to the small bowel's ability to act as a conduit for the products of digestion and the avoidance of obstruction. The morphometric analysis has given results very comparable to the stretch analysis. Much of the histologic morphometric analysis is automated and does not require the "wet bench" efforts of the stretch analysis. Moreover, the stretch analysis can only be applied to an organ that has a lumen that allows it to be placed over the pins of the stretch apparatus. This feature restricts us to

Table 1: Relation of a Predicted Incidence of Enteropathy (stained area greater than or equal to 8.2%) vs. an Observed Incidence of Enteropathy (i.e., any of the other assays positive for enteropathy)

Incidence of Enteropathy Predicted / Incidence of Enteropathy Observed	Positive	Negative
Positive	15	9
Negative	5	14

hollow organs with a more or less cylindric lumen. Even the bladder cannot be analyzed in this fashion since its lumen is not a cylinder and since the ureteric and urethral orifices do not lend themselves to being placed over the pins of the stretch meter.

We are presently in the process of applying this morphometric analysis to the other abdominal and pelvic organs harvested from our study animals and mounted as histologic specimens stained with trichrome. We hope to be able to demonstrate a positive protective effect of HBO_2 on these organs as well. Additional further study may involve other stains including immunohistochemical stains with monoclonal antibodies to collagen or specific collagen subtypes.

The results here are consistent with a radioprotective effect by HBO_2 delivered 7 wk after the completion of irradiation. As a radioprotector, the ability of HBO_2 to prevent the expression of delayed radiation injury well after the completion of the radiation course may represent an important advantage over the known chemical radioprotectors which must be given simultaneously with the radiation and which may protect tumor as well as normal tissues.

In addition, the methodology reported here should have application as a measure of the effectiveness of any radioprotector applied to delayed radiation injuries in animal studies.

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