Targeting Microparticles to Select Tissue via Radiation-Induced Upregulation of Endothelial Cell Adhesion Molecules

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Purpose. Certain endothelial cell adhesion molecules are up regulated in tissue that has been irradiated for therapeutic purposes. This up-regulation of adhesion molecules provides a potential avenue for targeting drugs to select tissues.

Methods. Microspheres were coated with a mAb to ICAM-1 and the level of adhesion of the anti-ICAM-1 microspheres to irradiated tissue *in vitro* and *in vivo* was quantified.

Results. Under *in vitro* flow conditions, the number of adherent microspheres on irradiated HUVEC was 4.8 ± 0.9 times that of control; the adhesion of anti-ICAM-1 microspheres on irradiated HUVEC could be enhanced by more than 170% in the presence of RBC (20% hematocrit) in the medium. *In vivo* in a rat cranial window model, the number of adherent anti-ICAM-1 microspheres in locally irradiated cerebral tissue was 8 and 13 times that of IgG microspheres at 24 h and 48 h post-irradiation, respectively and returned to baseline 7 days post-irradiation. In locally irradiated animals, the number of adhering microspheres in unirradiated tissue remained at the basal level.

Conclusions. Radiation-induced up-regulation of endothelial cell adhesion molecules may be exploited to target drugs and/or genes to select segments of the endothelium.

KEY WORDS: targeted drug delivery; adhesion molecules; radiation therapy; microcirculation.

INTRODUCTION

Radiotherapy is used to treat a variety of diseases including cancer, arteriovenous malformations (AVM), macular degeneration, and intimal hyperplasia. In most cases, using modern clinical radiotherapeutic techniques, radiation damage can be limited to a core of diseased tissue and the immediate normal tissue surrounding it. Similarly, it would be ideal for a pharmaceutical agent and/or a gene to be delivered preferentially to the diseased tissue and not to healthy tissue.

It is now well established that exposure of normal and diseased tissue to irradiation causes increases in microvascular permeability (1,2) and leukocyte infiltration of the tissues

(3–5) and that the microvasculature of tissue exposed to ionizing radiation is significantly altered. These alterations include the up-regulation of certain endothelial cell adhesion molecules (e.g., ICAM-1, E-selectin) on the lumenal surface of the endothelium (6–8). Although the issue of which endothelial cell adhesion molecules are expressed in response to radiation is currently under intense investigation, it is abundantly clear that the endothelial cell adhesion molecule profile is significantly altered in response to radiation. Even more noteworthy is a recent clinical study (6) reporting that both ICAM-1 and E-selectin were significantly up-regulated in oral mucosa of head and neck cancer patients treated with radiotherapy (30 to 60 Gy in 2 Gy daily fractions).

The radiation induced up-regulation of endothelial cell adhesion molecules provides the opportunity to target drugs to select tissue via a combination of radiation and ligand receptor drug targeting technology (9,10). In this manner, the radiation induced up-regulation of endothelial cell adhesion molecule(s) within the diseased tissue, which has been irradiated for therapeutic purposes, is used as a target to deliver drug carriers selectively to the site of disease. This approach has the added advantage of combining radiotherapy with drug and/or gene therapy in a local environment of increased microvascular permeability due to radiation exposure. For example, there is an increase in the blood-brain-barrier permeability after irradiation (1,2). Although this can lead to extravasation of blood proteins that may accelerate tissue injury, the increased permeability can enhance delivery of chemotherapeutic drugs across the blood-brain-barrier (2,11,12).

Recently Hallahan *et al.* (13) showed that peptides that bind to the β_3 integrin (a component of receptor GPIIb/IIIa, $\alpha_{2b}\beta_3$ that accumulates in the vessel lumen of irradiated tumors) can be used to deliver fluorochromes and radionuclides to irradiated tumors and observed a three to four fold increase in peptide binding in tumor vessels compared to adjacent normal tissue. In this study, we demonstrate the feasibility of targeting microparticles to select tissue via the up-regulation of adhesion molecules expressed on irradiated endothelial cells. In addition, we investigate the effect of hematocrit on this selective adhesion *in vitro*.

MATERIALS AND METHODS

Reagents

Human and rat serum were purchased from Irvine Scientific (Santa Ana, CA, USA) and bovine serum albumin (BSA) from Sigma (St. Louis, MO, USA). IL-1β was obtained from Calbiochem (La Jolla, CA, USA). Murine anti-rat ICAM-1 (1A29, IgG1) mAb was obtained from Pharmingen (Palo Alto, CA, USA). Murine anti-human ICAM-1 mAb (R6.5, IgG2a) was a generous gift from Boehringer Ingelheim (Ridgefield, CT, USA). Human IgG1,κ and mouse IgG1,κ were purchased from Sigma.

Non-fluorescent polystyrene particles for *in vitro* experiments (10 μm diameter, catalog #PS06N) were purchased from Bangs Laboratories (Fishers, IN, USA). Fluorescent 10 μm polystyrene microspheres for *in vitro* experiments (green: excitation 468nm, emission 508nm, catalog #G1000) were purchased from Duke Scientific (Palo Alto, CA, USA). Flueres-

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cent 2 μm microspheres (red: excitation 543nm, emission 612nm, catalog #R0200 & blue: excitation 412nm, emission 473nm, catalog #B0200) for *in vivo* experiments were purchased from Duke Scientific.

Generation of Ligand Coated Microspheres

Non-fluorescent and fluorescent polystyrene microspheres (10 μm diameter) for *in vitro* experiments and fluorescent 2 μm microspheres for *in vivo* experiments were coated with protein A via passive adsorption as described (14,15). To achieve this, the microspheres were incubated in a 0.1M NaHCO3, pH 9.2 buffer containing 300 μg/ml protein A (Zymed; San Fransico, CA, USA) at room temperature for over an hour. Following the adsorption, the microspheres were washed, incubated in a blocking buffer (Hank's balanced saline solution supplemented with 1% human or rat serum albumin), washed and incubated with human (for *in vitro* experiments) or rat (for *in vivo* experiments) mAb to ICAM-1 diluted in blocking buffer; matching microspheres were coated with IgG and used as control. The concentration of mAb and IgG in the coating solution used to generate the microspheres was greater than that needed to saturate the microspheres (as determined by FACS; not shown). After addition of the mAb or IgG, the microspheres were stored for a maximum of 7 days prior to use in an assay. Immediately prior to the assay, the microspheres were washed to remove unbound antibody or IgG. FACS analysis indicated that 96% of microspheres still had a similar level of ICAM-1 mAb on their surfaces at after 1 week of storage (data not shown). We suspect that the mAb to ICAM-1 and IgG does not dissociate from the microspheres during the 5 min *in vivo* assays because we have found in a previous study that an Fc construct, coupled to polystyrene microspheres via protein A, show little, if any, tendency to dissociate from the microspheres on incubation in mouse serum for 5 min at 37°C (16).

In Vitro Experiments

Human umbilical vein endothelial cells (HUVEC) were grown in 35 mm dishes and irradiated with a single dose of 10 Gy (gamma radiation, 4.2 Gy/min) as described before (7). Fluorescent polystyrene microspheres (10 μm in diameter, 5×10^5 /ml) bearing an antibody to human ICAM-1 (R6.5) or IgG (negative control) on their surface were perfused over the irradiated (IR) endothelial cells (48 h post-IR) in a parallel plate flow chamber as described before (7) at shear rates ranging from 70 to 260 s^{-1}. The number of firmly adherent microspheres to the endothelial monolayer was determined after 3 minutes of flow.

The presence of red blood cells has been shown to enhance the interaction of microspheres with the vessel wall *in vivo* and *in vitro* (17,18). Therefore, we tested the effects of the presence of red blood cells in the suspending media at two different concentrations (5% or 20% by volume) on the adhesion of microspheres to irradiated and control endothelial cells. Blood was obtained from guinea pigs by cardiac puncture. The supernatant was removed after centrifugation and red blood cells were washed three times in hepes buffer containing 1% BSA before being added to the media containing antibody bearing microspheres. The flow experiments were then carried out as described in the previous paragraph. Fluo-

rescent polystyrene microspheres (10 μm diameter) were used in all *in vitro* experiments where RBC was present in the suspending media. Non-Fluorescent polystyrene microspheres (10 μm diameter) were used in all other *in vitro* experiments.

In Vivo Experiments

The closed cranial window model was used as a model of normal tissue because the brain is a clinically relevant tissue in radiotherapy and pial vessels can be studied in an animal survival model using intravital microscopy techniques. Adhesion of microspheres to the brain microvasculature after testicular irradiation was also quantified to determine the specificity of microsphere adhesion to non-irradiated tissue in an irradiated animal. Conversely, adhesion of microspheres to the cremaster tissue microvasculature after local brain irradiation was quantified for the same purpose. The research adhered to the "Principles of Laboratory Animal Care" (NIH publication #85-23, revised in 1985).

In preparing the cranial window model, prior to surgery SD rats were anesthetized with an i.m. injection of 1 mL/kg of Ketaset (87mg ketamine/mL + 13 mg Xylazine/mL). Body temperature was maintained at approximately 37°C by convective heating. Animals were placed on a small animal stereotaxic frame. All surgical procedures were carried out under aseptic conditions. The animals were prepped with three applications of iodine to the shaved scalp before the initial incision was made. The scalp and tissue from a 1.5 × 1.5 cm area bilaterally over the parietal cortex was removed. Bleeding from soft tissue was controlled via heat cauterization as needed and the underlying fascia was blunt dissected. A rectangular cranial window extending from the coronal to the lambdoid sutures centered on the sagittal suture was traced using a dental drill at low speed. Care was taken to avoid frictional heat created by drilling for extended periods in any one area. Once the window was sufficiently drilled out, the flap of bone was gently removed with forceps and the underlying tissue washed with repeated applications of sterile artificial cerebrospinal fluid (ACSF). Slight bleeding from bridging vessels was allowed to clot without intervention. From this point on, all manipulations to the brain were carried out under a layer of sterile ACSF. The dura was punctured with a 30-gauge needle and the tissue excised with microdissecting scissors, with great care taken not to make contact with the underlying brain tissue. Superficial bleeding was allowed to stop without intervention and the tissue was irrigated regularly with sterile ACSF. A glass plate, resting on the bone surrounding the cranial window, was glued to the surrounding bone, using cyanocrylate glue. After recovery from anesthesia windowed animals were returned to the animal facilities and were given one week to recover from surgery.

In preparing the cremaster muscle for intravital microscopic observations (19) prior to surgery animals were anesthetized with i.m. injection of 1 mL/kg of Ketaset (87 mg ketamine/mL + 13 mg Xylazine/mL). Body temperature was maintained at approximately 37°C by convective heating. Animals were intubated, catheterized (left femoral vein), and placed on a surgical board where the right cremaster muscle was pinned as a flat sheet with minimal disruption to the tissue. Preparations were maintained at a temperature of 36 ± 0.5°C and superfused at a rate of 5 ml/min with a bicarbonate buffered salt solution equilibrated with 5%CO^2–95% N^2.

Prior to irradiation animals were sedated with an i.m. injection of 0.5 mL/kg of Ketaset (87 mg ketamine/mL + 13 mg Xylazine/mL). A local single dose (10 Gy) of radiation was delivered locally to the brain or to the testicles at a rate of 2 Gy/min using a Siemens MD-2 linear accelerator (6 MV X-rays) located at the St. Jude Children's Research Hospital. A collimator, 1.5 cm in diameter and normally used for human stereotactic radiosurgery, was used to localize the radiation dose. Tissue equivalent bolus was placed above and below the tissue to establish electronic equilibrium and to insure the prescribed dose is delivered uniformly to the tissue.

At different time points before and after irradiation, 2 μm fluorescent polystyrene microspheres bearing rat anti-ICAM-1 or IgG (1×10^8 microspheres/animal) on their surface were injected via tail vein. Dual color fluorescent microscopy was used to quantify the level of adhesion of anti-ICAM-1 and IgG (control) bearing microspheres to the cerebral or cremaster venules before and after irradiation. The number of anti-ICAM-1 and IgG bearing microspheres adhering to all the vessels in the tissue being observed was counted and the results are presented as the "number of adherent microspheres/mm^2" of tissue. Data was taken while quickly alternating the two sets of fluorescent filters thus visualizing one set of microspheres at the time. There was no significant difference between the level of adhesion of red and blue 2 μm fluorescent microspheres (bearing rat anti-ICAM-1 or IgG) to the venules, i.e., changing the color of the beads did not have an effect of the level of adhesion (data not shown).

Statistics

Data are presented as mean ± standard error of the mean (Mean ± SEM). Analysis of variance (ANOVA) was used to determine the effects of irradiation on drug carrier interaction with irradiated endothelium or irradiated tissue. A multiple comparison procedure (Fisher's least significant difference, LSD) was used to discriminate among the means. The paired t test was used to compare the interaction of the microspheres with endothelium in each microvessel before and after irradiation. Differences were considered to be statistically significant if $p < 0.05$.

RESULTS

In vitro adhesion of anti-ICAM-1 microspheres to irradiated endothelial cells is higher than their adhesion to normal endothelial cells over a large range of shear values and the level of adhesion can be modulated by hematocrit.

A flow chamber was used to study the interaction of microparticles bearing human anti-ICAM-1 ligands on their surfaces with irradiated endothelial cells under shear flow conditions. All *in vitro* experiments were carried out 48 h post-irradiation because previously we have reported that ICAM-1 is maximally upregulated on HUVEC at 48 h post-irradiation (7). Under shear flow conditions (200 s^{-1}) the number of adherent anti-ICAM-1 microspheres on 48 h post-irradiated HUVEC was significantly ($p < 0.01$) higher than control, see Fig. 1. ICAM-1 is expressed at a basal level on control cells (20) which explains the adhesion of anti-ICAM-1 microspheres to control cells. We next perfused the anti-ICAM-1 microspheres over 48 h post-irradiated HUVEC and
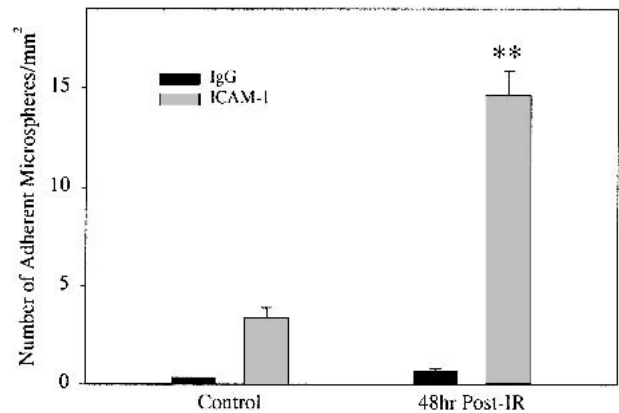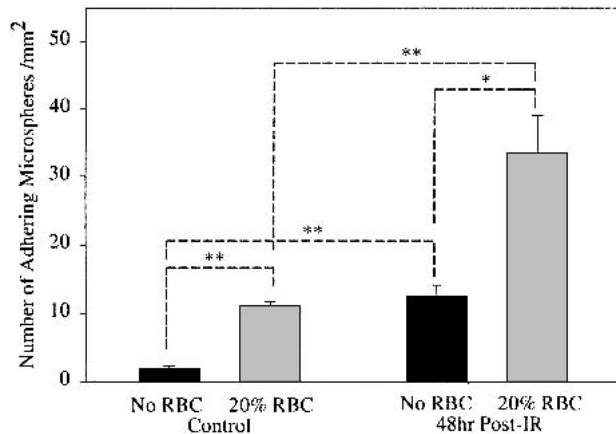

Fig. 1. Under shear flow conditions (shear rate of 200 s^{-1}) number (Mean ± SEM) of adherent anti-ICAM-1 microspheres on 48h post-irradiated HUVEC was significantly (ANOVA, ** $p < 0.01$) different from that of control. $N = 5$ experiments per group, ** $p < 0.01$.

control HUVEC at various shear rates starting at a relatively high shear rate, 260 s^{-1}, down to a relatively low shear rate, 70 s^{-1}. We allowed the microspheres to collect on the HUVEC at each shear rate for a set period of time and then reduced the shear to the next shear rate. The cumulative number of adherent anti-ICAM-1 microspheres was determined at the end of each period. As shown in Fig. 2, over the entire range of shear rates tested, the cumulative number of adherent anti-ICAM-1 microspheres was greater on 48 h post-irradiated HUVEC relative to control HUVEC. Interestingly, the cumulative difference in adhesion observed between the 48 h post-irradiated HUVEC relative to control HUVEC at the lower two shear rates appeared to be due to the difference in adhesion between the two HUVEC at the higher two shear rates. The shear rate values used in these *in vitro* experiments (70 to 260 s^{-1}) correlate with *in vivo* blood flow in post-capillary venules of both normal and irradiated tissue (19,21).

The adhesion of anti-ICAM-1 microspheres on irradiated HUVEC, as well as control HUVEC, could be enhanced by more than 170% in the presence of RBC (20% hematocrit) in the medium, see Fig. 3. Even at 5% hematocrit there was
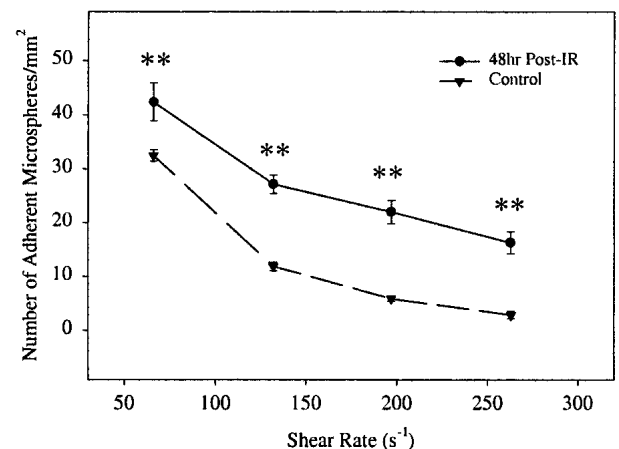
Fig. 2. The cumulative number of adherent anti-ICAM-1 microspheres on irradiated HUVEC was larger than that of control over the range of shear values used in these experiments, $N = 6$–7 experiments per group, ** $p < 0.01$ indicate significant differences as compared to the corresponding control group.

Fig. 3. Adhesion of anti-ICAM-1 microspheres on irradiated HUVEC under shear flow conditions (shear rate of 200 s^{-1}), as well as control HUVEC, was significantly enhanced in the presence of RBC (20% hematocrit) in the medium. Dotted lines indicate significant differences between corresponding groups. Mean ± SEM, N = 5–8 experiment per group, * p < 0.05, ** p < 0.01.

a significant increase in the number of microspheres adhering to irradiated, but not control, HUVEC (data not shown). These results demonstrate that anti-ICAM-1 coated microspheres exhibit augmented adhesion to irradiated HUVEC *in vitro* in a large range of shear values and that this adhesion can be further enhanced by increasing red blood cell concentration in the suspending medium.

In vivo adhesion of ligand bearing microspheres to irradiated tissue is upregulated as compared to control, and these microspheres are preferentially targeted to irradiated tissue.

A rat closed cranial window model was used to study the interaction of microparticles bearing rat anti-ICAM-1 ligands on their surfaces with irradiated cerebral tissue *in vivo*. Our results indicate that in the irradiated tissue a large number of anti-ICAM-1 coated microspheres adhere to the cerebral vessel walls (Fig. 4A), whereas very few IgG coated microspheres adhere to the walls of the same vessels (Fig. 4B). The number of adherent anti-ICAM-1 microspheres in irradiated cerebral tissue was 8 times that of IgG coated microspheres at 24 h after irradiation. Two days after irradiation, the number of adherent anti-ICAM-1 microspheres increased to more than 13 times that of IgG coated microspheres, see Fig. 5A basal level of anti-ICAM-1 coated microsphere adhesion to non-irradiated tissue is expected because a low basal level of ICAM-1 is constitutively expressed on endothelium in all tissue under control conditions (22). The number of adherent anti-ICAM-1 microspheres to the brain microvasculature returned to the control level 7 days post-irradiation. Injection (i.v.) of the 500 μg of antibody to rat ICAM-1 (1A29, IgG1) 15 min prior to the experiment completely blocked adhesion of anti-ICAM-1 coated microsphere 48 h post-irradiation (data not shown); this experiment was not carried out at other time points post-irradiation due to the prohibitive cost of the ICAM-1 mAb.

Adhesion of microspheres to (non-irradiated) brain microvasculature after testicular irradiation was also quantified to determine the specificity of microsphere adhesion to only the locally irradiated tissue. Irradiating the cremaster muscle did not significantly increase the adhesion of microspheres to the (non-irradiated) brain microvasculature, see Fig. 6A.
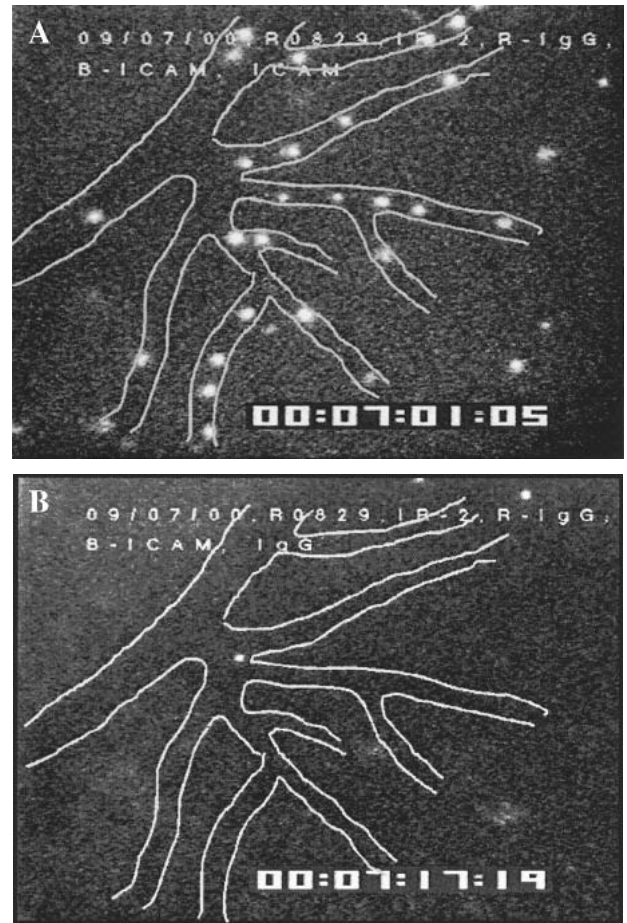

Fig. 4. *In vivo* in a rat closed cranial window model, a large number of anti-ICAM-1 coated microspheres adhere to the vessel walls (Panel A), whereas very few IgG coated microspheres adhere to the walls of the same vessels (Panel B). The outlines of the vessels have been digitally superimposed on the darkfield image.

Along the same line, our results indicate that at 48 h post-irradiation while irradiating the cremaster resulted in a significant (p < 0.01) increase in the adhesion of microspheres to the cremaster microvasculature, irradiating the brain did not significantly increase the adhesion of microspheres to the (nonirradiated) cremaster microvasculature, Fig. 6B.

These results demonstrate that the adhesion of ligand bearing microspheres to the microvasculature is upregulated post irradiation and that this targeted delivery mechanism is specific to irradiated tissue.

DISCUSSION

In this study we have shown that microparticles can be selectively and preferentially targeted to irradiated endothelial cells *in vitro* and irradiated microvasculature *in vivo* via radiation-induced upregulation of endothelial cell adhesion molecules. Our *in vitro* findings indicate that adhesion of these microspheres to the irradiated endothelium is significantly larger than control and can be significantly modulated by the concentration of red blood cells in the suspending media. Our *in vivo* findings indicate that this targeting mechanism is very much localized to the irradiated tissue in that the basal level of adhesion of antibody-bearing microspheres to
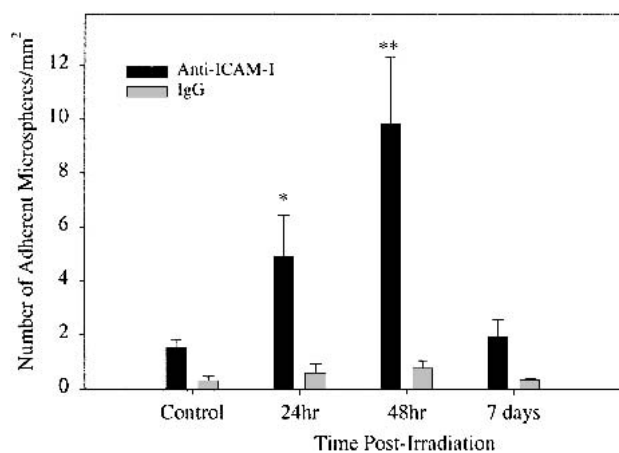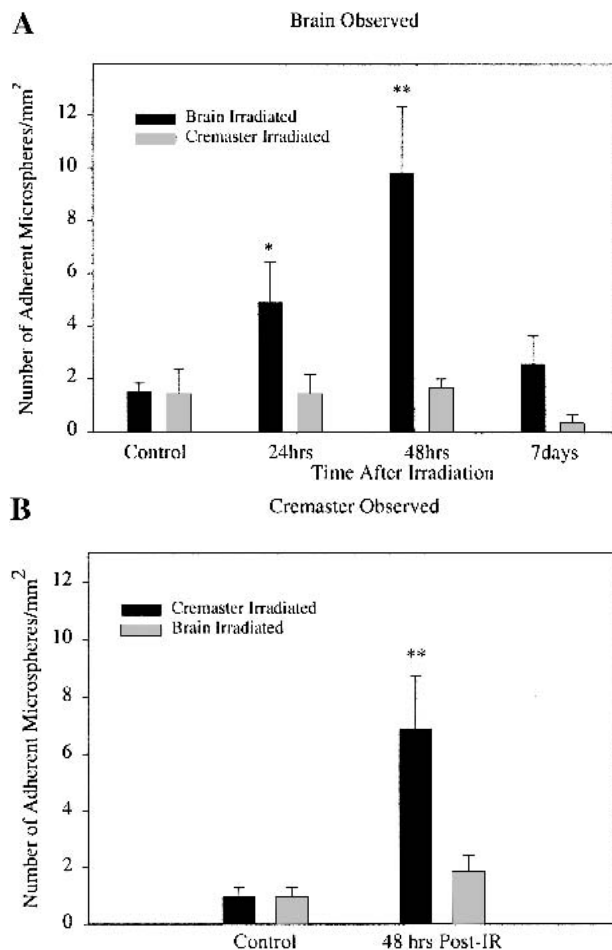
Fig. 5. Number of adherent anti-ICAM-1 microspheres in irradiated cerebral tissue increased significantly post-irradiation, peaked at two days after irradiation and returned to its control level 7 days post-irradiation. Mean ± SEM, N = 6–8 animals per experiments, * p < 0.05, ** p < 0.01 indicate significant differences as compared to control.

Fig. 6. Irradiating the cremaster muscle did not significantly increase the adhesion of microspheres to the (non-irradiated) brain microvasculature, A. Consistently, 48 hours post-irradiation while irradiating the cremaster resulted in a significant (p < 0.01) increase in the adhesion of microspheres to the cremaster microvasculature, irradiating the brain did not significantly increase the adhesion of microspheres to the (non-irradiated) cremaster microvasculature, B. Mean ± SEM, N = 4–5 animals per experiments, * p < 0.05, ** p < 0.01 indicate significant differences as compared to control.

nonirradiated tissue in locally irradiated animals was not found significantly different from that in control (non-irradiated) animals.

Presence of blood cells, especially red blood cells, which enhance the interaction of particles and leukocytes with the endothelium (17,18) may prove beneficial for this approach. On the other hand, various adhesion molecules not only exist on endothelial cells but also on some blood cells. For example ICAM-1 exists on both endothelial cells and leukocytes (23). This may be a potential problem in that these blood cells may compete with the endothelium for binding to the drug carrier and the drug carrier could be delivered to the non-target cell (e.g. the leukocyte). Our initial findings indicate that the enhancing effects of presence of red cell may be dominant, i.e. interaction of anti-ICAM-1 bearing microspheres with endothelium *in vivo* is significantly higher than that seen *in vitro* in the absence of RBC. In addition, we did not observe any adhesive interactions between leukocytes and anti-ICAM-1 bearing microspheres *in vivo*.

We would like to note that making comparisons between the *in vitro* and *in vivo* data is quite difficult and was not the main point of our study. In particular, we point out that we have used two different sized microspheres for the *in vitro* (10 µm) and *in vivo* (2 µm) studies and it is quite clear that particle size can affect adhesion under flow (15). Our motivation for using the 10-µm microspheres *in vitro* was that such sized microspheres are easy to manipulate and detect in adhesion assays. Our motivation for using the 2 µm microspheres *in vivo* was that they are most likely the largest sized particles that will fit through the smaller vessels of the microcirculation yet large enough to easily detect in the *in vivo* assays. Even though there may not be a one-to-one correspondence between adhesion levels obtained *in vivo* and in vitro, the overall findings in both systems clearly indicate that microparticles can be targeted to irradiated tissue via the up-regulation of adhesion molecules.

This work has important implications for emerging drug delivery strategies that seek to target drugs, genes or other particles of interest (e.g., contrast agents) to select segments of the endothelium (9,10,16,24,25). There is an increasing effort to generate biodegradable drug carriers that target select segments of the endothelium. In particular, we have previously shown that biodegradable particles coated with a humanized mAb to E- and P-selectin exhibit limited selective adhesion to endothelial expressed E-selectin and transfected cell lines expresseing P-selectin (10). Recently a biodegradable polymer has been described that rolls on surfaces coated with soluble E- and P-selectin (26). The methodology developed here can be extended to preferentially target clinically relevant particles (e.g., liposomes, biodegradable polymeric drug carriers) to tissue that has been irradiated for therapeutic purposes. This approach also has the added advantage of targeting pharmaceutical agents to an irradiated tissue site that often experiences increased vascular permeability (1,2).

We started the development of this drug targeting technology with microspheres bearing mAbs to ICAM-1 due to the fact that there is very convincing evidence that ICAM-1 is up-regulated by ionizing radiation (4,7,27–29) and the easy

availability of this antibody. However, a basal level of ICAM-1 is constitutively expressed on endothelium in all tissue under control conditions (22) as well as on leukocytes and other adhesion molecules such as E-selectin may be more suited for this approach. The methodology developed in this study could be easily extended to include the development of particles bearing antibodies to E-selectin and other adhesion molecules of interest as additional data on the up-regulation of adhesion molecules on the surface of various irradiated endothelial cells becomes available.

Tumor microvasculature and its endothelial cells may vary from normal tissue endothelial cells in many ways (30). However, many of the adhesion molecules that are expressed in normal tissue in response to ionizing radiation are also expressed in tumors (8). In addition, other adhesion molecules and native molecular markers may be present on a tumor (31,32) that could provide additional targets for drug delivery. For example, drug carrying particles targeting both adhesion molecules (upregulated on tumors and its surrounding normal tissue in response to irradiation) and native molecular markers present in a tumor may provide an even more powerful approach than targeting adhesion molecules alone.

In summary, our data suggest that radiation-induced up-regulation of endothelial cell adhesion molecules may be exploited to target drugs and/or genes to select segments of the endothelium. Currently we are developing pharmacologically relevant biodegradable drug carriers that can be targeted to irradiated tissue clinically (10).

ACKNOWLEDGMENT

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