

Differences in ICAM-1 and TNF- α expression between large single fraction and fractionated irradiation in mouse brain

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(Received 15 July 2002; accepted 9 January 2003)

Abstract.

Purpose: To elucidate the brain molecular response to irradiation. The expression of the intercellular adhesion molecule (ICAM-1) and tumour necrosis factor- α (TNF- α) in the mouse brain was compared after single-dose and fractionated whole-brain irradiation. **Materials and methods:** Mice received a single dose of 2, 10 or 20 Gy or a fractionated dose (2 Gy day⁻¹) of 10, 20 or 40 Gy. ICAM-1, and TNF- α mRNA expression were quantified by the highly sensitive real-time polymerase chain reaction technique. Expression of ICAM-1 protein was quantified by dual-labelled monoclonal antibody assay.

Results: After a 20-Gy single dose, there was an increase in ICAM-1 and TNF- α mRNA levels (14- and 11-fold, respectively) as well as a significant increase in the level of ICAM-1 protein ($p=0.0243$). The expression of ICAM-1 and TNF- α mRNA increased at the end of the 40-Gy fractionated regimen (3.55- and 2.30-fold, respectively).

Conclusions: The molecular response of the brain to single-dose irradiation was rapid, while its response to fractionated irradiation was slow. This finding is consistent with clinical observations and could be of use when designing strategies to mitigate radiation sequelae.

1. Introduction

High-dose single-fraction and fractionated radiotherapy (RT) regimens are instrumental to the successful treatment of brain tumours. However, the effectiveness of RT is limited by collateral damage to normal brain tissue. Radiation causes a molecular response in the brain (Hong *et al.* 1995). This response, which is marked by increased expression of adhesion molecules and cytokines (Chiang *et al.* 1993b, Acker *et al.* 1998, Quarmby *et al.* 2000), may play an important role in the pathogenesis of radiation sequelae and/or tissue recovery in patients who are treated with cranial RT. Increased expression of adhesion molecules may also provide a unique opportunity to target drugs and/or genes to tissue that has been irradiated

for therapeutic purposes (Hallahan *et al.* 2001, Kiani *et al.* 2002).

Expression of ICAM-1, which is constitutively expressed in the brain by endothelial cells, microglia and astrocytes, is upregulated by inflammatory stimuli. The upregulation of this inducible cell surface glycoprotein plays a role in a variety of disease processes and conditions, predominantly by interfering with normal immune function (van de Stolpe and van der Saag 1996). *In vitro* studies have shown that ICAM-1 plays an important role in the increased adhesion of leukocytes to irradiated endothelium (Gaugler *et al.* 1997, Hallahan and Virudachalam 1997, Prabhakarparandian *et al.* 2001). Upregulation of ICAM-1 expression has also been observed in a number of organs in response to ionizing radiation (Quarmby *et al.* 2000). Limited information is available about the effect of irradiation on ICAM-1 expression in the brain (Chiang *et al.* 1993a, Kyrkanides *et al.* 1999, Kiani *et al.* 2002) especially in response to fractionated doses.

TNF- α has a broad spectrum of biological responses including cell adhesion, tissue injury, acute and chronic inflammation, the release of other inflammatory cytokines, and cell death (Bazzoni and Beutler 1996). In the brain, TNF- α is expressed by endothelial cells and almost all brain cells and it has been associated with the injured brain tissue in Alzheimer's disease and in ischaemic brain injury (Mattson *et al.* 1997, Meistrell *et al.* 1997). However, Bruce *et al.* (1996) showed that TNF- α mediates cerebroprotection in an induced cerebral ischaemia model. This beneficial role is dependent on the expression levels of the molecule (Meistrell *et al.* 1997). Radiation induces a TNF- α response in the brain (Hong *et al.* 1995, Chiang *et al.* 1997). Besides its usual implication in brain tissue damage, TNF- α has been shown to have a possible protective role against the development of late radiation sequelae (Daigle *et al.* 2001).

Most preclinical studies of radiation-induced ICAM-1 and TNF- α expression in normal tissues rely on single-dose irradiation, and they often use higher doses than are usually administered in clinical settings. Although some studies have investigated the dose-effect

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relationship in normal tissues (Giri *et al.* 1985, Kamiryo *et al.* 1996), none to date has investigated the possible differences in the molecular responses induced by single-dose and fractionated RT regimens in normal brain tissue. Such information should help to explain the mechanisms that allow normal tissues to tolerate or modulate the effects of radiation.

Real-time PCR was used to quantify ICAM-1 and TNF- α mRNA after single and fractionated doses of ionizing radiation. It has been shown to provide sensitive, precise and reproducible quantification of specific mRNAs that are expressed at low levels (Wu *et al.* 1994, Rubin and Staddon 1999). The double-stranded DNA-binding dye SYBR Green, which undergoes marked fluorescence enhancement upon binding to double-stranded DNA, was used to monitor PCR product formation (Wu *et al.* 1993, Morrison *et al.* 1998). The dual-labelled monoclonal antibody (mAb) assay has been used to quantify the constitutive and induced expression of ICAM-1 and E- and P-selectins in murine models of various disease states (Henninger *et al.* 1997, Lundberg *et al.* 2000). This assay was used to compare quantitatively the expression of ICAM-1 protein in the brains of mice after single-dose and fractionated irradiation of the brain.

2. Materials and methods

2.1. Animals

All experiments used 6–7-week-old male C57Bl/6j mice (Harlan Laboratories, Frederick, MD, USA) maintained on normal rodent chow. The animals were anaesthetized with an intramuscular injection of a mixture of 10 mg kg⁻¹ xylazine (Rompun Mobay Corp., Shawnee, KS, USA) and 87 mg kg⁻¹ ketamine hydrochloride (Ketaset, Aveco Co., Fort Dodge, IA, USA). A 6-MV linear accelerator (Siemens Primus, Concord, CA, USA) was used for all experiments. Radiation treatment was delivered by using a 3.5-cm diameter collimator in a single-field configuration, and a tissue-equivalent material was placed above the mouse's head to establish electronic equilibrium and ensure uniform delivery of the prescribed dose to the brain. The dose rate for all experiments was 3 Gy min⁻¹. All protocols were approved by the Animal Care and Use Committee of St Jude Children's Research Hospital and followed the policy guidelines of the National Institutes of Health for the humane care and use of laboratory animals.

2.2. Real-time PCR assay of ICAM-1 and TNF- α Mrna

Six groups of four animals each received a single dose of 20-Gy whole-brain irradiation. ICAM-1 and

TNF- α mRNA in brain tissue were assayed at a specified time after irradiation (2, 4, 8, 12, 24 or 48 h) in each group. A control group ($n=4$) of unirradiated animals underwent ICAM-1 and TNF- α measurements. In three groups of four animals each that received 2-Gy whole-brain irradiation, ICAM-1 and TNF- α mRNA were assayed 2, 4 or 8 h after irradiation. Three groups of three animals underwent fractionated irradiation (10, 20 or 40 Gy). Each group received 2-Gy whole-brain irradiation daily for 5 days of each week up to the specified total dose. Four animals were used in a sham experiment (different than the control group). The animals were anaesthetized daily, 5 days/week, for 4 weeks. They were placed in the treatment room but were not irradiated. ICAM-1 and TNF- α were assayed 2 h after the last dose was given. A control group of five unirradiated animals underwent identical assay.

2.2.1. RNA extraction and real-time PCR protocol. RNA was extracted from brain tissue by using RNeasy B (Tel-test, Inc., Friendswood, TX, USA). Residual DNA was removed by treatment with 2 units DNase I (Ambion, Inc., Austin, TX, USA) at 37°C for 30 min; the enzyme was inactivated by incubation at room temperature for 2 min. Total RNA was quantified by a GeneQuant Pro spectrophotometer (Amersham Pharmacia Biotechnologies, Piscataway, NJ, USA). A TaqMan Reverse Transcription Reagents kit (PE Applied Biosystems, Foster City, CA, USA) was used to reverse-transcribe 2 μ g RNA. Real-time PCR was performed in the PE Biosystems GeneAmp 7700 sequence detection system by using SYBR Green PCR Master Mix as recommended by the manufacturer. Each reaction contained 25 μ l 2 \times SYBR Green PCR Master Mix. Primers for GAPDH (final concentration 50 nM), ICAM-1 and TNF- α (final concentration 150 nM) were added. A typical reaction contained 3 μ l cDNA in a total reaction volume of 50 μ l. The protocol consisted of heating at 50°C for 2 min and at 95°C for 10 min, followed by 40 cycles of heating at 95°C for 15 s and at 60°C for 1 min. The primers for GAPDH, ICAM-1 and TNF- α (table 1) were designed by using the Primer Express[®] software (PE Applied Biosystems). All these primer pairs produced amplicons less than 150 bp in length.

2.2.2. Normalization of PCR values by those obtained for the housekeeping gene GAPDH. The quantification method uses the log phase of the reaction. This method assumes that the input amounts of RNA for all samples are equal (Allt and Lawrenson 2000). The gene encoding GAPDH, which is present in

Table 1. Primers and amplification products.

Gene	Primer sequence (forward and reverse)	Accession number of gene sequence	Amplification product	
			bp (<i>n</i>)	T_M ($^{\circ}$ C)
GAPDH	5'-TGT GTC CGT CGT GGA TCT GA 5'-TCA AGA AGG TGG TGA AGC	M32599.1	77	82
ICAM-1	5'-CGC AAG TCC AAT TCA CAC TGA 5'-CTT TTG CTC TGC CGC TCT G	M31585.1	72	81
TNF- α	5'-GTA CCT TGT CTA CTC CCA GGT TCT CT5'-GTG TGG GTG AGG AGC ACG TA	M12305.8	69	83

bp, base pairs; T_M , approximate melting temperature.

constant amounts in all samples, was used as an internal reference. In each experiment, the GAPDH mRNA was quantified in an aliquot of each sample by quantitative PCR. To normalize for inefficiencies in cDNA synthesis and RNA input amounts, copy numbers obtained for GAPDH were subtracted from the ICAM-1 and TNF- α copy numbers (see equation 1).

2.2.3. *Analysis.* The fluorescence signal ΔR_n was calculated by the PE Biosystems 7700 sequence detection system software as:

$$\Delta R_n = (R_n^+) - (R_n^-),$$

where R_n^+ is the fluorescence signal of the product at any given time and R_n^- is the fluorescence signal of the baseline emission during cycles 3–15. The log ΔR_n fluorescence signal was then plotted against the cycle number and an arbitrary point was chosen at the midpoint of the curve. C_t was defined as the cycle number at which log ΔR_n crossed this threshold. The fold change in ICAM-1 and TNF- α cDNA (target gene) relative to GAPDH cDNA was determined using:

$$\text{Fold change} = 2^{-\Delta\Delta C_t}, \quad (1)$$

where $\Delta\Delta C_t = (C_{t\text{Target}} - C_{t\text{GAPDH}})_{\text{Time } x} - (C_{t\text{Target}} - C_{t\text{GAPDH}})_{\text{Time } 0}$. Time x is any time point, and time 0 is the expression of the gene in the unirradiated (control) state (Rubin *et al.* 1999). The mean (\pm SE) values for the ICAM-1 and TNF- α genes in each group were plotted as a function of time and dose.

2.3. Dual-labelled monoclonal antibody assay for ICAM-1 protein

ICAM-1 protein was assayed in four groups of animals at the following times after a single dose of 20-Gy whole-brain irradiation: 24 h ($n=5$), 48 h (4), 72 h (4) and 192 h (i.e. 8 days) (3). Control levels of ICAM-1 protein were measured in a group ($n=5$) of unirradiated animals. Because the peak response occurred 48 h after irradiation, ICAM-1 expression

was measured only at 48 h in the subsequent single-fraction experiment (10 Gy, $n=3$). Three additional groups of four animals each underwent fractionated irradiation (total dose 10, 20 or 40 Gy). Each group received 2-Gy whole-brain irradiation daily for 5 days of each week up to the specified total dose. ICAM-1 protein was assayed 48 h after the last dose was given. A group of five unirradiated animals underwent ICAM-1 measurement as controls.

Purified endotoxin-free mAb to mouse CD54 (ICAM-1) clone 3E2B (Endogen, Woburn, MA, USA) was used as the binding antibody for *in vivo* ligation of ICAM-1. The non-binding isotype-matched antibody clone A19-3 (Pharmingen, San Diego, CA, USA) was used in conjunction with the binding antibody to quantify ICAM-1 expression. The iodogen method (Fraker and Speck, Jr 1978) was used to radiolabel the binding mAb with iodine 125 (I^{125}) and the non-binding mAb with iodine 131 (I^{131}) (DuPont NEN, Boston, MA, USA). Previous studies have shown that the iodogen method of mAb labelling does not interfere with mAb function (Panes *et al.* 1995).

2.3.1. *Measurement of ICAM-1 protein expression.* The jugular vein and carotid artery of each anaesthetized mouse were cannulated with PE10 and PE50 tubing (Becton Dickinson, Sparks, MD, USA), respectively. A mixture of 10 μ g I^{125} -labelled ICAM-1-binding mAb (to detect ICAM-1 on the surface of endothelial, astrocytic and glial cells), 40 μ g cold unlabelled anti-ICAM-1 mAb (to saturate the cell surface receptors) and an appropriate amount of the I^{131} -labelled non-binding mAb A19-3 (400 000–600 000 cpm, to eliminate the influence of non-specific binding to cells) was retrograde administered through the jugular vein catheter (total volume 200 μ l). A blood sample was exsanguinated from the carotid artery 5 min after initial injection of the mAb mixture. The animals were then given 40 units heparin and exsanguinated through the carotid artery catheter while a bicarbonate-buffer saline solution was infused through the venous catheter. The brain tissue

was then harvested and weighed. A Cobra Automated Gamma Counting System (Packard Instruments, Meriden, CT, USA) measured the ^{125}I and ^{131}I radioactivity in tissue samples and in 50- μl samples of serum; values were corrected for background activity and spillover. Each mAb measurement was expressed as the percentage of the injected dose (%ID) of antibody g^{-1} dry-weight tissue. The following equation was used to calculate ICAM-1 expression:

$$\text{Expression } (\mu\text{g mAb/g}) = \left(\frac{I^{125} \% \text{ ID/g (dry weight tissue)}}{I^{125} \% \text{ ID injected}} - \frac{I^{131} \% \text{ ID/g (dry weight tissue)}}{I^{131} \% \text{ ID injected}} \right) \times \text{total injected mAb } (\mu\text{g})/100 \quad (2)$$

Finally, as a positive control for our assay and for the inducibility of ICAM-1 protein expression, 50 μg *Salmonella abortus equi* lipopolysaccharide (LPS) (Sigma Chemical Co., St Louis, MO, USA) was administered intraperitoneally to each mouse in a group of five. Twenty-four hours later, ICAM-1 expression in the brain and heart was measured by using the methods described above.

2.4. Data analysis

The mean (\pm SE) values for the ICAM-1 protein in each group were plotted as a function of time and dose. Analysis of variance with Fisher's test for significance was used to compare the ICAM-1 kinetics in the 20-Gy single dose, and the fractionated dose groups. In the 10-Gy single dose group, the two groups (control and 48 h) were compared by the Student's *t*-test.

3. Results

3.1. Expression of TNF- α and ICAM-1 mRNA

Because SYBR Green can bind to primer dimers formed non-specifically during all PCR reactions, a melting-curve analysis was carried out to test for the specificity of the primer pairs listed in table 1. It verified the specificity of the primer pairs selected (data not shown).

ICAM-1 and TNF- α mRNA expression were measured in mouse brain 2, 4, 8, 12, 24 and 48 h after a single 20-Gy dose of whole-brain irradiation. The greatest acute response (a 14-fold increase in the expression of ICAM-1 mRNA) was seen 2 h after irradiation. Similarly, TNF- α mRNA expression peaked at 2 h after irradiation (an 11-fold increase) (figure 1). In mice that completed a 2-week regimen of fractionated irradiation (total dose 20 Gy), ICAM-1 mRNA

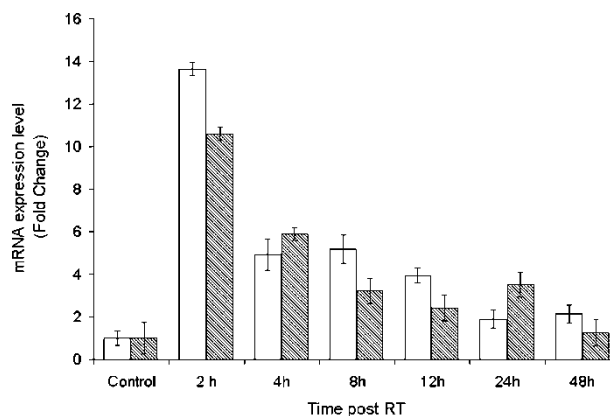


Figure 1. Acute-phase kinetics of ICAM-1 and TNF- α mRNA expression in mouse brain after a single 20-Gy dose of whole-brain irradiation. Expression of ICAM-1 and TNF- α mRNA increased 14- and 11-fold, respectively, 2 h after irradiation. Each value is the mean \pm SE of four measurements. \square , ICAM-1; \blacksquare , TNF- α .

levels were near those of controls 2 h after the final fraction was delivered (figure 2). After 4 weeks of fractionated irradiation (40-Gy total dose), ICAM-1 mRNA expression was 3.55-fold greater than that in controls 2 h after the final fraction was delivered. Again, TNF- α mRNA levels showed a 2.3-fold increase at the end of the fractionated regimen (40-Gy total dose) with almost no change during the 4-week treatment period (figure 2).

To study the kinetics of ICAM-1 and TNF- α mRNA expression after low-dose irradiation, assays were performed 2, 4 and 8 h after irradiation with a single dose of 2 Gy. ICAM-1 mRNA levels were slightly higher than those of controls at any time point (1.44-fold at 2 h, 1.41-fold at 4 h, 1.12-fold at 8 h). This was not the case for the TNF- α mRNA levels, which were higher than control only at the 2 h time

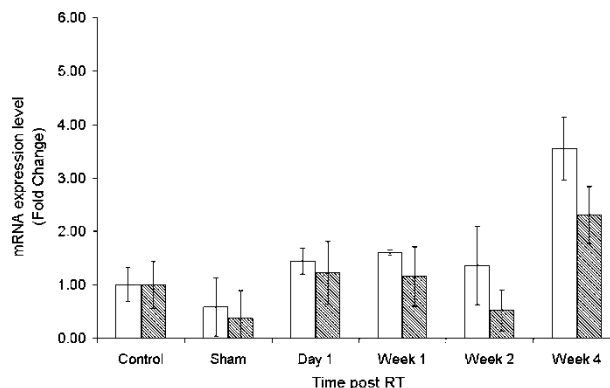


Figure 2. Expression of ICAM-1 and TNF- α mRNA 2 h after delivery of the final fraction of a 2 (day 1)-, 10 (week 1)-, 20 (week 2)- or 40 (week 4)-Gy total dose of radiation (2 Gy day $^{-1}$). Each value is the mean \pm SE of three to four measurements. \square , ICAM-1; \blacksquare , TNF- α .

point (1.22-fold). The 2h value is included in figure 2 as the 'day 1' time point for comparison with the fractionated levels of ICAM-1 and TNF- α .

To study the effect of repeated doses of anaesthesia and animal handling on our fractionated results, a sham experiment was carried out on a group of four animals that received no irradiation but which were anaesthetized daily for 4 weeks. No significant difference was found between the sham and control groups in the levels of ICAM-1 and TNF- α mRNA (figure 2).

3.2. Expression of ICAM-1 protein after single-fraction irradiation

Expression of ICAM-1 protein at 48h ($0.274 \pm 0.06 \mu\text{g mAb g}^{-1}$) after irradiation of the whole mouse brain with a single dose of 20 Gy was significantly greater than the baseline value (0.099 ± 0.01 , $p = 0.011$). ICAM-1 protein expression was increased at all time points investigated (24 h, $p = 0.201$; 72 h, $p = 0.656$; 8 days, $p = 0.527$) (figure 3).

Because maximal expression occurred at this time point, ICAM-1 protein expression was measured 48 h after a single dose of 10 Gy; protein expression ($0.231 \pm 0.06 \mu\text{g mAb g}^{-1}$) was significantly increased above baseline ($p = 0.002$) at 48 h post-RT (data not shown). These results obtained with the dual-labelled mAb assay were consistent with earlier *in vitro* findings using a flow chamber assay (Prabhakarbandian *et al.* 2001) and with *in vivo* findings using anti ICAM-1 coated microspheres (Kiani *et al.* 2002).

3.3. Expression of ICAM-1 protein after fractionated irradiation

ICAM-1 expression was measured 48 h after the completion of fractionated courses (2 Gy day^{-1}) of

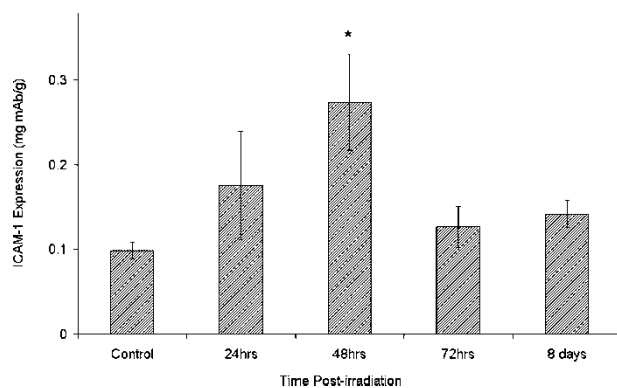


Figure 3. Kinetics of ICAM-1 protein expression in mouse brain after a single 20-Gy dose of whole-brain irradiation. Expression reached a peak 48 h after irradiation ($*p = 0.011$). Each value is the mean \pm SE of four measurements.

irradiation at total doses of 10, 20 and 40 Gy. ICAM-1 expression in the brains of mice given 10 Gy ($0.067 \pm 0.01 \mu\text{g mAb g}^{-1}$), 20 Gy ($0.109 \pm 0.01 \mu\text{g mAb g}^{-1}$) or 40 Gy ($0.078 \pm 0.02 \mu\text{g mAb g}^{-1}$) whole-brain irradiation did not differ significantly from that in controls ($0.096 \pm 0.02 \mu\text{g mAb g}^{-1}$, $p > 0.386$, 0.708 and 0.602) (figure 4). Forty-eight hours after the final fraction was delivered, expression of ICAM-1 protein was significantly greater in the mice given a single dose of 10 Gy than in those given 10 Gy by fractionated irradiation ($p = 0.004$). It was also significantly greater in the mice given 20 Gy as a single dose than in those given 20-Gy fractionated irradiation ($p = 0.029$) (figure 5). The effects of fractionated irradiation on the molecular expression of ICAM-1 in the brain have not been measured directly, but the

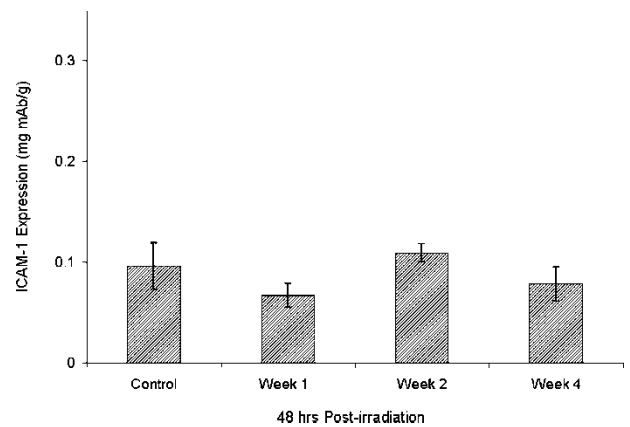


Figure 4. ICAM-1 protein expression in mouse brain 48 h after delivery of the final fraction after 1 (10 Gy), 2 (20 Gy) and 4 weeks (40 Gy) of fractionated irradiation (2 Gy day^{-1}). No significant increase in ICAM-1 protein was observed at any time point. Each value is the mean \pm SE of three to four measurements.

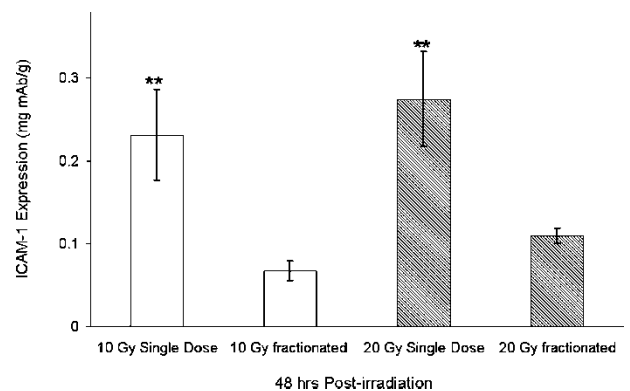


Figure 5. Comparison of the effect of the single-dose and fractionated regimens on the expression of ICAM-1 protein in mouse brain. There was a significant difference in ICAM-1 expression 48 h after irradiation at total doses of 10 and 20 Gy ($*p = 0.002$, $**p = 0.029$). Each value is the mean \pm SE of three to four measurements.

present findings are consistent with well-documented clinical observations of the disparate acute side-effects of the two types of regimens.

To confirm the detectability and inducibility of ICAM-1 expression, constitutive ICAM-1 protein expression and that induced by administration of LPS in the brain and heart muscle of five mice were measured. A large difference was observed between the two organs in the level of constitutive ICAM-1 protein expression (brain $0.099 \pm 0.01 \mu\text{g mAb g}^{-1}$, heart $1.449 \pm 0.34 \mu\text{g mAb g}^{-1}$). Twenty-four hours after administration of LPS, ICAM-1 expression was significantly increased in both organs (brain $0.266 \pm 0.03 \mu\text{g mAb g}^{-1}$, $p=0.008$; heart, $3.943 \pm 0.33 \mu\text{g mAb g}^{-1}$, $p=0.015$). These results agree with Henninger *et al.* (1997) who observed large tissue-to-tissue differences in ICAM-1 expression.

4. Discussion

The effect of fractionated irradiation on the molecular expression of ICAM-1 and TNF- α in the brain using highly sensitive and quantitative techniques of real-time RT-PCR and dual mAb has been measured for the first time. The difference in ICAM-1 and TNF- α expression between high single doses and fractionated doses of radiation has also been delineated. The results indicate that single and fractionated doses of radiation caused significantly different acute-phase expression of ICAM-1 and TNF- α in normal mouse brain. Forty-eight hours after irradiation was completed, significantly less ICAM-1 protein was detected in the brain tissues of mice that received fractionated doses than in the brain tissues of mice that received equivalent single-dose irradiation. A single dose of 20 Gy induced an acute molecular response, as measured by expression of ICAM-1 protein and mRNA that peaked 48 and 2 h, respectively, after irradiation, and TNF- α mRNA that peaked at 2 h after irradiation. Fractionated irradiation at a total dose of 40 Gy caused an increase in ICAM-1 and TNF- α mRNA, although it did not significantly increase ICAM-1 protein expression. However, a single dose of 2 Gy did not significantly increase the expression of either ICAM-1 or TNF- α mRNA. These findings may help to explain why fractionated irradiation causes fewer acute side-effects in normal tissue than does equivalent single-dose irradiation. Although not directly indicated by our data, the almost equal levels of ICAM-1 protein after irradiation with 10- and 20-Gy single doses (figure 5) seems to exclude any dose-response to single doses of radiation above 10 Gy. Similarly, our fractionated data do not suggest any dose-response due to fractionated doses less than 40 Gy (figure 2).

Little information is available about the acute-phase molecular response of the brain and especially following fractionated dose regimens. Hong *et al.* (1995) reported increased expression of ICAM-1 mRNA in mice 4 h after irradiation of the midbrain with single doses as low as 2 Gy. However, it is unclear whether or not the increase was significant. Hong *et al.* also report a threshold dose level of 7 Gy for TNF- α mRNA expression (measured 4 h after irradiation) below which they did not detect the messenger molecule. Although their data showed an increase in ICAM-1 and TNF- α mRNA levels following a single dose of 25 Gy, they do not report a specific fold increase. Chiang *et al.* (1997) reported an increased level of TNF- α mRNA from 4 h to 6 months following a dose of 25 Gy to the brain. The same study also reported that ICAM-1 mRNA levels peaked at 4 h following the same radiation dose and then declined steadily thereafter. Olschowka *et al.* (1997) detected increased ICAM-1 immunohistochemical staining in both the endothelial cells and astrocytes throughout the irradiated brain 4 h to 7 days after administration of a single fraction of 25 Gy. These findings are consistent with those of our experiments, in which ICAM-1 protein expression peaked 48 h after a dose of 20 Gy and ICAM-1 and TNF- α mRNA increased 14- and 11-fold, respectively, 2 h after the same dose. Also, ICAM-1 and TNF- α mRNA expression increased (although not significantly) 1.44- and 1.22-fold, respectively, 2 h after treatment with 2 Gy. Note that our measurements were carried out with two highly sensitive and quantitative techniques, real-time PCR and the dual-labelled mAb assay.

It has been shown that irradiation with a dose of 10 Gy causes a significant increase in the adhesion of anti-ICAM-1 coated microspheres to the cerebral microvasculature (Kiani *et al.* 2002). The increased microsphere-endothelial interaction was increased starting at 24 h after irradiation, peaked at 48 h after irradiation and returned to the control level 7 days after irradiation. This agrees with the findings shown in figure 3. Our *in vitro* findings (Prabhakarpanthian *et al.* 2001) also indicate that ICAM-1 upregulation on endothelial cells increases at 24 h and peaks at 48 h after irradiation.

Our fractionated dose experiments (figure 4) could also be viewed as a delivered dose measurement with ICAM-1 expression measured (always 48 h after the last fraction) at 10, 20 and 40 Gy after the start of irradiation. When viewed in this manner, our findings show that the expression of ICAM-1 protein was not upregulated at any time during a 4-week, 40-Gy fractionated regimen. Expression of both ICAM-1 and TNF- α mRNA, which were measured 2 h after each week's last fraction was delivered, showed no

increase until the entire 4-week regimen was completed (a total dose of 40 Gy), at which time there was an increase in both molecule's levels.

The difference in the levels of molecular response between the single-dose and fractionated experiments might be indicative of a duality in the functional role of these molecules. The measured increase, immediately following the high, single doses of irradiation, could be part of the tissue damage cytokines cascade (Michalowski 1994, Tofilon and Fike 2000). On the other hand, the slower and cumulative rise in these molecules following 4 weeks of fractionated radiation could be part of the tissue recovery process. Even though the present study does not provide direct evidence for such interpretation, TNF- α has a beneficial effect in arresting long-term radiation sequelae and in reducing the infarct size following ischaemic brain injury (Bruce *et al.* 1996, Daigle *et al.* 2001). These reported beneficial effects are dependent on the expressed levels of the cytokine, with higher levels implicated in tissue damage and cell death. The role of ICAM-1 in the brain response to radiation is still to be elucidated. As others have suggested, ICAM-1 levels in the brain may increase in response to a complex cascade of events, such as elevated levels of cytokines, breakdown of the blood-brain barrier (Rubin *et al.* 1994), upregulated expression of other inflammatory molecules or neurological degeneration (Merrill and Murphy 1997).

In summary, this *in vivo* quantitative study has provided evidence for an early-phase molecular response to large, single doses of radiation and that this response is attenuated by dose fractionation. This finding is consistent with clinical observations, but it does not account for the long-term side-effects of fractionated dose regimens. Our detection of increased ICAM-1 and TNF- α mRNA at the end of a 40-Gy fractionated regimen may indicate the early phase of a low-level response after irradiation. To address this further, we are in the process of determining the molecular response to fractionated doses at longer time intervals. It is speculated that the clinical sequelae of such an effect might emerge long after the end of treatment. We are currently seeking to elucidate the intermediate and long-term side-effects of fractionated dose regimens on the brain.

Acknowledgements

The work was supported in part by a Cancer Center Support CORE Grant P30 CA 21765 and by the American Lebanese Syrian Associated Charities (ALSAC). The authors thank Sharon Naron, ELS, for editorial assistance.

References

- ACKER, J. C., MARKS, L. B., SPENCER, D. P., YANG, W., AVERY, M. A., DODGE, R. K., ROSNER, G. L. and DEWHIRST, M. W., 1998, Serial *in vivo* observations of cerebral vasculature after treatment with a large single fraction of radiation. *Radiation Research*, **149**, 350–359.
- ALLT, G. and LAWRENSON, J. G., 2000, The blood–nerve barrier: enzymes, transporters and receptors — a comparison with the blood–brain barrier. *Brain Research Bulletin*, **52**, 1–12.
- BAZZONI, F. and BEUTLER, B., 1996, The tumor necrosis factor ligand and receptor families. *New England Journal of Medicine*, **334**, 1717–1725.
- BRUCE, A. J., BOLING, W., KINDY, M. S., PESCHON, J., KRAEMER, P. J., CARPENTER, M. K., HOLTSBERG, F. W. and MATTSON, M. P., 1996, Altered neuronal and microglial responses to excitotoxic and ischemic brain injury in mice lacking TNF receptors. *Nature Medicine*, **2**, 788–794.
- CHIANG, C. S., HONG, J. H., STALDER, A., SUN, J. R., WITHERS, H. R. and MCBRIDE, W. H., 1997, Delayed molecular responses to brain irradiation. *International Journal of Radiation Biology*, **72**, 45–53.
- CHIANG, C. S., MCBRIDE, W. H. and WITHERS, H. R., 1993a, Myelin-associated changes in mouse brain following irradiation. *Radiotherapy Oncology*, **27**, 229–236.
- CHIANG, C. S., MCBRIDE, W. H. and WITHERS, H. R., 1993b, Radiation-induced astrocytic and microglial responses in mouse brain. *Radiotherapy Oncology*, **29**, 60–68.
- DAIGLE, J. L., HONG, J. H., CHIANG, C. S. and MCBRIDE, W. H., 2001, The role of tumor necrosis factor signaling pathways in the response of murine brain to irradiation. *Cancer Research*, **61**, 8859–8865.
- FRAKER, P. J. and SPECK, J. C. JR., 1978, Protein and cell membrane iodinations with a sparingly soluble chloroamide, 1,3,4,6-tetrachloro-3a,6a-diphrenylglycoluril. *Biochemical and Biophysical Research Communications*, **80**, 849–857.
- GAUGLER, M. H., SQUIBAN, C., VAN DER MEEREN, A., BERTHO, J. M., VANDAMME, M. and MOUTHON, M. A., 1997, Late and persistent up-regulation of intercellular adhesion molecule-1 (ICAM-1) expression by ionizing radiation in human endothelial cells *in vitro*. *International Journal of Radiation Biology*, **72**, 201–209.
- GIRI, P. G., KIMLER, B. F., GIRI, U. P., COX, G. G. and REDDY, E. K., 1985, Comparison of single, fractionated and hyperfractionated irradiation on the development of normal tissue damage in rat lung. *International Journal of Radiation Oncology, Biology, Physics*, **11**, 527–534.
- HALLAHAN, D. E., QU, S., GENG, L., CMELAK, A., CHAKRAVARTHY, A., MARTIN, W., SCARFONE, C. and GIORGIO, T., 2001, Radiation-mediated control of drug delivery. *American Journal of Clinical Oncology*, **24**, 473–480.
- HALLAHAN, D. E. and VIRUDACHALAM, S., 1997, Ionizing radiation mediates expression of cell adhesion molecules in distinct histological patterns within the lung. *Cancer Research*, **57**, 2096–2099.
- HENNINGER, D. D., PANES, J., EPPHIMER, M., RUSSELL, J., GERRITSEN, M., ANDERSON, D. C. and GRANGER, D. N., 1997, Cytokine-induced VCAM-1 and ICAM-1 expression in different organs of the mouse. *Journal of Immunology*, **158**, 1825–1832.
- HONG, J. H., CHIANG, C. S., CAMPBELL, I. L., SUN, J. R., WITHERS, H. R. and MCBRIDE, W. H., 1995, Induction of acute phase gene expression by brain irradiation. *International Journal of Radiation Oncology, Biology, Physics*, **33**, 619–626.
- KAMIRYO, T., KASSELL, N. F., THAI, Q. A., LOPES, M. B., LEE, K. S.

- and STEINER, L., 1996, Histological changes in the normal rat brain after gamma irradiation. *Acta Neurochirurgica*, **138**, 451–459.
- KIANI, M. F., YUAN, H., CHEN, X., SMITH, L., GABER, M. W. and GOETZ, D. J., 2002, Targeting microparticles to select tissue via radiation-induced upregulation of endothelial cell adhesion molecules. *Pharmaceutical Research*, **19**, 1317–1322.
- KYRKANIDES, S., OLSCHOWKA, J. A., WILLIAMS, J. P., HANSEN, J. T. and O'BANION, M. K., 1999, TNF alpha and IL-1beta mediate intercellular adhesion molecule-1 induction via microglia-astrocyte interaction in CNS radiation injury. *Journal of Neuroimmunology*, **95**, 95–106.
- LUNDBERG, A. H., GRANGER, D. N., RUSSELL, J., SABEK, O., HENRY, J., GABER, L., KOTB, M. and GABER, A. O., 2000, Quantitative measurement of P- and E-selectin adhesion molecules in acute pancreatitis: correlation with distant organ injury. *Annals of Surgery*, **231**, 213–222.
- MATTSON, M. P., BARGER, S. W., FURUKAWA, K., BRUCE, A. J., WYSS-CORAY, T., MARK, R. J. and MUCKE, L., 1997, Cellular signaling roles of TGF beta, TNF alpha and beta APP in brain injury responses and Alzheimer's disease. *Brain Research Brain Research Review*, **23**, 47–61.
- MEISTRELL, M. E., III., BOTCHKINA, G. I., WANG, H., DI SANTO, E., COCKROFT, K. M., BLOOM, O., VISHNUBHAKAT, J. M., GHEZZI, P. and TRACEY, K. J., 1997, Tumor necrosis factor is a brain damaging cytokine in cerebral ischemia. *Shock*, **8**, 341–348.
- MERRILL, J. E. and MURPHY, S. P., 1997, Inflammatory events at the blood brain barrier: regulation of adhesion molecules, cytokines, and chemokines by reactive nitrogen and oxygen species. *Brain Behavior, and Immunology*, **11**, 245–263.
- MICHALOWSKI, A. S., 1994, On radiation damage to normal tissues and its treatment. II. Anti-inflammatory drugs. *Acta Oncologica*, **33**, 139–157.
- MORRISON, T. B., WEIS, J. J. and WITTWER, C. T., 1998, Quantification of low-copy transcripts by continuous SYBR Green I monitoring during amplification. *Biotechniques*, **24**, 954–962.
- OLSCHOWKA, J. A., KYRKANIDES, S., HARVEY, B. K., O'BANION, M. K., WILLIAMS, J. P., RUBIN, P. and HANSEN, J. T., 1997, ICAM-1 induction in the mouse CNS following irradiation. *Brain Behavior, and Immunology*, **11**, 273–285.
- PANES, J., PERRY, M. A., ANDERSON, D. C., MANNING, A., LEONE, B., CEPINSKAS, G., ROSENBLUM, C. L., MIYASAKA, M., KVIETYS, P. R. and GRANGER, D. N., 1995, Regional differences in constitutive and induced ICAM-1 expression *in vivo*. *American Journal of Physiology*, **269**, H1955–H1964.
- PRABHAKARPANDIAN, B., GOETZ, D. J., SWERLICK, R. A., CHEN, X. and KIANI, M. F., 2001, Expression and functional significance of adhesion molecules on cultured endothelial cells in response to ionizing radiation. *Microcirculation*, **8**, 355–364.
- QUARMBY, S., HUNTER, R. D. and KUMAR, S., 2000, Irradiation induced expression of CD31, ICAM-1 and VCAM-1 in human microvascular endothelial cells. *Anticancer Research*, **20**, 3375–3381.
- RUBIN, L. L. and STADDON, J. M., 1999, The cell biology of the blood–brain barrier. *Annual Review of Neuroscience*, **22**, 11–28.
- RUBIN, P., GASH, D. M., HANSEN, J. T., NELSON, D. F. and WILLIAMS, J. P., 1994, Disruption of the blood–brain barrier as the primary effect of CNS irradiation. *Radiotherapy Oncology*, **31**, 51–60.
- TOFILON, P. J. and FIKE, J. R., 2000, The radioresponse of the central nervous system: a dynamic process. *Radiation Research*, **153**, 357–370.
- VAN DE STOLPE, A. and VAN DER SAAG, P. T., 1996, Intercellular adhesion molecule-1. *Journal of Molecular Medicine*, **74**, 13–33.
- WU, N. Z., KLITZMAN, B., ROSNER, G., NEEDHAM, D. and DEWHIRST, M. W., 1993, Measurement of material extravasation in microvascular networks using fluorescence video-microscopy. *Microvascular Research*, **46**, 231–253.
- WU, N. Z., ROSS, B. A., GULLEDGE, C., KLITZMAN, B., DODGE, R. and DEWHIRST, M. W., 1994, Differences in leucocyte-endothelium interactions between normal and adenocarcinoma bearing tissues in response to radiation. *British Journal of Cancer*, **69**, 883–889.

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