UAB LIBRARIES
The University of Alabama at Birmingham

University of Alabama at Birmingham
UAB Digital Commons

All ETDs from UAB

UAB Theses & Dissertations

2014

IL-8 is necessary and sufficient for X-ray radiation enhanced endothelial adhesion.

Stephen Kyle Babitz
University of Alabama at Birmingham

Follow this and additional works at: https://digitalcommons.library.uab.edu/etd-collection

Part of the Medical Sciences Commons

Recommended Citation

Babitz, Stephen Kyle, "IL-8 is necessary and sufficient for X-ray radiation enhanced endothelial adhesion." (2014). *All ETDs from UAB*. 1071.
https://digitalcommons.library.uab.edu/etd-collection/1071

This content has been accepted for inclusion by an authorized administrator of the UAB Digital Commons, and is provided as a free open access item. All inquiries regarding this item or the UAB Digital Commons should be directed to the UAB Libraries Office of Scholarly Communication.

IL-8 IS NECESSARY AND SUFFICIENT FOR X-RAY RADIATION ENHANCED ENDOTHELIAL ADHESION

by

STEPHEN BABITZ

RALPH SANDERSON, CHAIR
DANIEL BULLARD
JANUSZ KABAROWSKI
DENNIS KUCIK, MENTOR
JOANNE MURPHY-ULLRICH
RAKESH PATEL
CHRISTOPHER WILLEY

THESIS

Submitted to the graduate faculty of The University of Alabama at Birmingham,
in partial fulfillment of the requirements for the degree of
Master of Science

BIRMINGHAM, ALABAMA

2014

IL-8 IS NECESSARY AND SUFFICIENT FOR X-RAY RADIATION ENHANCED ENDOTHELIAL ADHESION

STEPHEN BABITZ

MOLECULAR AND CELLULAR PATHOLOGY

ABSTRACT

Ionizing radiation (IR) is a potent inflammatory stimulus to the human body. In the vasculature, inflammation is a major contributing factor for atherosclerosis. In addition, radiation from several sources has been linked with increased risk for multiple cardiovascular complications.

A key compnent of radiation associated inflammation is an increase in the adhesiveness of the endothelium which leads to a pathogenic accumulation of leukocytes in the vascular wall. This is one of the initial steps in vascular inflammation and leads to a number of adverse complications such as heart disease and stroke. The molecular mechanisms behind radiaton enhanced endothelial adhesion have not been completely delineated. Our previous studies with HAECs interogating the molecular mechanism responsible for enhanced endothelial adhesiveness have shown that chemokine signaling is required.

Further investigation using luminex technology and in-vitro laminar flow adhesion assays showed that IL-8 was in fact the chemokine responsible. 15 Gy X-ray radiation greatly induced secretion of IL-8 in an acute fashion. In addition inhibiting IL-8 completely ablated the adhesion enhanced by radiation. In addition treating unirradiated endothelial cells with both recombinant human IL-8 and media harvested from irradiated endothelial cells was able to replicate the adhesion effect seen after directly irradiating endothelial

cells. Our studies also show that heparan sulfate glycosaminoglycan chains, which are responsible for chemokine presentation, present on the endothelial cell surface are also critical for radiation enhanced endothelial adhesion. Due to the soluble nature of IL-8 it is likely that heparan sulfate binds IL-8 secreted in response to radiation and then presents IL-8 to the chemokine receptors on flowing monocytic cells.

Keywords: radiation, IL-8, adhesion, chemokines

ACKNOWLEDGMENTS

Dr. Ralph Sanderson and his lab members for reagents and consultation. Dr. Chad Steele and Chad Dunaway for conducting the luminex assays. Funding from NASA/NSBRI.

TABLE OF CONTENTS

Page

ABSTRACT ii

ACKNOWLEDGMENTS iv

LIST OF TABLES v

LIST OF FIGURES vi

BACKGROUND 1

METHODS 7

RESULTS 10

DISCUSSION 23

FUTURE PERSPECTIVES 27

LIST OF REFERENCES 29

LIST OF TABLES

Tables *Page*

1 Effects of radiation on secretion of multiple chemokines determined by Luminex assay 15

LIST OF FIGURES

Figure		Page
1	Heparin Addition to HAECs inhibits radiation enhanced adhesion but not TNF-α induced adhesion.	10
2	Enzymatic digestion of endothelial heparan sulfate by Hep3 inhibits radiation enhanced adhesion, but not TNF-α induced adhesion	12
3	Addition of media from 15 Gy irradiated cells (without TNF-α) to unirradiated cells 24 hours prior to adhesion is sufficient for enhanced adhesion.	13
4	IL-8 is secreted by HAECs in a time dependent manner consistent with an acute response to radiation.	17-18
5	Anti- lL-8 Fab fragments inhibit radiation enhanced adhesion, but not TNF-α induced adhesion.	20
6	Addition of rhIL-8 to unirradiated cells 24 hours prior to adhesion assay is sufficient for enhanced adhesion.	21
7	Diagram showing treatment timeline HAECs undergo prior to adhesion assay	22

BACKGROUND

Radiation is a potent pathological stimulus that can increase risk for cardiovascular disease and stroke. Studying the atomic bomb survivors has shown that cardiovascular disease is prominent cause of death (Shimizu et al. BMJ, 2010). Furthermore patients who received radiation therapy for head and neck cancer have been shown to have increased risk for stroke (Cheng et al. Am J Surg 1999). Exposure to radiation, such as workers exposed to occupational sources of radiation, before 1950 has shown increased cardiovascular disease mortality (Hauptmann et al. Am J Epidem. 2003). Many of the adverse cardiovascular consequences resulting from radiation exposure can be traced back to increased inflammatory response. The molecular mechanisms underlying the pathogenesis of radiation induced vascular inflammation, however, are not well understood.

Atherosclerosis is an inflammatory disease characterized by the progression of fatty streaks on the arterial vasculature into plaques that can cause an acute clinical event (Berliner et al. Circulation 1995). A combination of the secretion of oxidative products from cells of the artery wall and transport of LDL across the endothelium into the subendothelial space results in oxidized lipoproteins becoming trapped in the vascular wall (Simionescu et al. AJP 1986, Witzum Lancet 1994). Accumulation of oxidized lipoproteins in the arterial wall causes activation of pro-inflammatory transcription

factors such as NFκB (Liao et al. JCI 1993). Activation of NFκB then results in up regulation of pro-inflammatory cytokines such as TNF-α which leads to initial development of fatty streaks via monocyte infiltration (Liao et al. JCI 1994).

Atherosclerotic lesion formation is a process by which circulating monocytes infiltrate the vascular wall of larger arteries and form dangerous lipid-rich lesions called plaques. These fatty lesions commonly form in the aorta due to the presence of pro-inflammatory stimuli such as irregular blood flow, resulting in uptake and subendothelial accumulation of cholesterol laden plasma lipoproteins such as LDL (Sima et al. Cell Tissue Res. 2009). This pro-inflammatory environment results in leukocytes in general and monocytes in particular to adhere to the shear stress activated endothelium of the aorta. Once these cells adhere to the endothelium they then migrate through the endothelium to form fatty lesions. These lesions grow over time due to further activation of the endothelium as a result of pro-inflammatory cytokines secreted by monocytes in the lesion (Libby et al. J Am Coll. Cardiol. 2009). Eventually these lesions will become enlarged and form atherosclerotic plaques that consume a large cross sectional area of the vascular lumen.

The monocytic infiltration cascade involves multiple steps, with one of the most critical being the arrest, or firm adhesion, of the monocytes to the endothelial cell monolayer of the vascular wall, allowing subsequent transmigration into the vascular intima. The initial step of the cascade is selectin mediated rolling, where endothelial selectins bind their respective ligands on circulating monocytes (Springer. Cell 1994). The critical step then occurs when rolling monocytes arrest upon activated integrins binding the endothelial

integrin ligands ICAM-1 and VCAM-1. Particularly important are the interactions between the $\alpha_M\beta_2$ and $\alpha_L\beta_2$ integrins which bind ICAM-1, and $\alpha_4\beta_1$ integrin which binds VCAM-1 (Langer et al. JCMM 2009). In atherosclerotic mouse models, deficiency in ICAM-1 has shown to be atheroprotective (Collins et al. J Exp Med. 2000). Integrin mediated firm adhesion then allows subsequent transendothelial migration into the vascular intima. These adhesion molecules are prominent on activated EC's, along with a robust profile of chemokines which serve to trigger integrin activation. Once cells have migrated into the intima they serve to cause a further inflammatory reaction due to the secretion of pro-inflammatory cytokines.

We have previously shown that chemokines are important to dose-dependent radiation enhanced endothelial adhesiveness under flow (Khaled et al. Rad. Res. 2011). In these studies it was shown that 15 Gy X-ray irradiation at 24 hours post irradiation of endothelial cells had the maximal effect on radiation enhanced endothelial adhesion. Under these condtions expression of the endothelial integrin ligands ICAM-1 and VCAM-1 was unchanged. However individual blockade of α_4, β_1, and β_2 integrins on THP-1 cells did abrogate the adhesion enhanced by radiation indicating that all of these integrins are necessary for radiation enhanced adhesion, but not TNF-α induced adhesion. TNF-α was used to stimulate HAECs six hours prior to adhesion assays to stimulate the expression of endotheial adhesion molecules such as selectins and integrin ligands. This experimental design is standard for flow adhesion assays to induce baseline adhesiveness of endothelial cells. In our assays radiation enhances adhesion when the radiation occurs 18 hours prior to TNF-α treatment. We have shown radiation enhanced adhesion was attenuated by treating THP-1 cells with the G-protein Coupled Receptor (GPCR)

inhibitor Pertusssis Toxin (PTX). PTX causes the G_i subunit of the G proteins present in THP-1 cells to remain locked in the inactive state and unable to facilitate downstream signaling (Burns. Microbio Sci. 1988). PTX treatment reduced the adhesion of THP-1 cells to irradiated endothelial cells to TNF-α induced adhesion levels, but had no effect on adhesion of THP-1 cells to unirradiated endothelial cells treated with TNF-α. Since these GPCRs are critical for endothelial chemokine signalling to activate leukocyte integrins, it was concluded that this radiation enhanced adhesion was dependent on chemokine signaling.

Our previous studies indicated only that chemokine signaling was responsible for radiation enhanced endothelial adhesiveness. We therefore sought to delineate which chemokine, or chemokines, in particular acted to mediate this effect. Previous studies by other groups have indicated non-aortic endothelial cells produce IL-8 in response to radiation and TNF-α, however they did not attribute a functional outcome as a result (Meeren et al. Mediators of Inflammation 1997). IL-8 mediates endothelial adhesion through the G-protein coupled receptors (GPCRs) CXCR1 and CXCR2 that consequently engage CD11a/CD18 and CD11b/CD18 integrin activation (Leong et al. Pro. Science 1997, Heit et al. JCS 2005). This activation is primarily facilitated by G-protein signaling leading to Phospholipase C (PLC) signaling and subsequent IP_3-induced release of intracellular calcium (Lomakina et al. Cell Mol Bioeng. 2010). Loss of the IL-8 receptor CXCR2 expression *in vivo* decreases atherosclerotic development (Boisvert et al, JCI 1998). Although IL-8 was originally thought to play a role in neutrophil adhesion, IL-8 has also been shown to increase monocyte adhesion to activated endothelial cells (Schwartz et al. JCI 1994). In fact IL-8 has been shown to mediate adhesion of the THP-1

monocytic cell line used in this study to linoleic acid treated HAECs by utilizing neutralizing antibodies to reduce adhesion (Matesanz et al. JNB 2012). IL-8 was also shown to be a factor in decoy receptor 3 (DcR3) enhanced endothelial adhesiveness to U937 and THP-1 cells (Yang et al. J Immu. 2005). It is also important to note multiple forms of IL-8 can be found in supernatant as a result of varying physiological and pathological conditions. For instance, the pro-inflammatory proteases plasmin and thrombin target serine residues in the N-terminus of the 77aa form of IL-8 for cleavage to produce truncated forms of IL-8. Others have shown that some of the truncated forms of IL-8 possess increased binding affinity for heparin and more actively engage CXCR1 and CXCR2 (Mortier et al. Plos one 2011).

Chemokines such as IL-8 are soluble proteins secreted from the cells and it is important to understand the process by which chemokines facilitate signaling from the endothelial cells to flowing monocytic cells thereby resulting in adhesion. Soluble chemokines require presentation by sulfated proteoglycan glycosaminoglycan (GAG) chains to interact with GPCR's (Proudfoot. Biochem. Soc. Trans. 2006). IL-8 immobilization on the cellular glycocalyx has been shown to induce the adhesion of leukocytes to the endothelium via CD11/CD18 (Carveth et al. BBRC 1989). Studies by the Esko and Fukuda groups have shown *in vivo* and *in vitro* that endothelial cells deficient in heparan sulfate, the predominant GAG in aortic endothelium, have decreased ability to present chemokines and subsequently show decreased leukocyte adhesion and trafficking (Bao et al. Cell 2010, Wang et al. Nature Immun. 2005). These studies showed that deficiencies in endothelial heparan sulfate biosynthesis pathway, Exostosin-1 (EXT1) and N-deacetylase/N-sulfotransferase 1 (NDST1) in particular, impaired endothelial chemokine

binding and presentation. In the endothelial cells lacking EXT1, the enzyme responsible for HS chain polymerization, Bao et al. showed that diminished lymphocyte homing was due to decreased chemokine presentation. NDST1 catalyzes the initial N-sulfation step of HS which has been shown to be important for adhesion and chemokine binding (Wang et al. Nature Immun. 2005). After NDST1 modification, the highly negatively charged HS is able to bind the positively charged C-terminus of the soluble chemokines. While sulfation is important for binding of IL-8 and other chemokines, recent studies have shown that 2-O-sulfation inhibits IL-8 binding and inactivation of the enzyme responsible for this modification enhances leukocyte binding (Axelsson et al. Blood. 2012). While the complexities of the interactions between chemokines and sulfated GAGs are not yet fully understood, our data suggest that it is possible for radiation to not only facilitate adhesion via chemokines, but also through modification of the proteoglycans present on the endothelial cell surface.

Because radiation can cause a number of different health consequences as a result of increased inflammation in the vasculature, heart attack and stroke in particular, the mechanism that causes inflammation in response to radiation is important. Adhesion of monocytic cells to vascular endothelium is a critical step in the inflammatory process that leads to adverse health consequences. We previously showed that X-ray radiation enhanced adhesion in a dose dependent manner and required chemokine signaling (Khaled et al. Rad. Res. 2011). To better understand the mechanism we need to identify the chemokine(s) involved and determine if the chemokine response to radiation is consistent with an acute or chronic response.

METHODS

Cells for experiments. Primary Human Aortic Endothelial Cells (HAECs) were purchased from Lonza, Walkerville, MD and shipped to UAB frozen at passage 3 and grown to passage 5, frozen and stored in liquid nitrogen. Cells were grown on fibronectin/gelatin coated tissue culture vessels in endothelial growth media with 2% FBS (with additional supplements) and used for experiments at passages 6 and 7. THP-1 cells were purchased from ATCC, Manassas, VA, and grown in RPMI with 10% FBS.

Irradiation at UAB. Confluent HAECs were irradiated with X-rays at 320 KeV in the UAB animal resource facility. Dose rates at 12.5 mA are approximately 2.7 Gy/min. 0 Gy (control) cells were similarly transported along with the cells that are being irradiated. Since maximal increase in adhesion occurred at 15 Gy, that dose was used for most of the studies. Cell growth media was changed immediately following radiation so that the irradiation of media does not affect cell viability.

Supernatant collection and ELISA. Endothelial cell culture supernatant was collected to quantify protein released into the supernatant at two hour intervals following irradiation. Cells that were irradiated 24 hours prior to media collection were also stimulated with TNF-α for 6 hours (with untreated controls) and protein in the supernatant was analyzed.

Luminex. Luminex kits (Millipore) will be used to identify the individual chemokines that are secreted in response to radiation. Kits were run using luminex instrumentation in collaboration with Chad Steele's lab. Supernatants analyzed by luminex were collected as detailed above.

In-vitro **Adhesion assay.** Adhesion assays were performed at 24 hours post irradiation using a custom built flow cell adhesion system. Endothelial media is removed and a flow chamber insert is inserted into 35mm cell culture dish in which endothelial cells have been grown to confluence. This combination forms a laminar flow chamber. Before the assay is conducted, all endothelial cells were stimulated with TNF-α (50 ng/ml) for 6 hours. This is the standard setup for flow chamber experiments to study adhesion under shear stress conditions.

THP-1 cells (ATCC, Manassas, VA) were injected into the flow chamber in serum free RPMI ($1x10^6$ cells/ml) using a syringe pump with flow rate set to achieve 1 $dyne/cm^2$ shear stress. Cells contacting the endothelium were viewed via bright field microscope transmitting to a CCD camera model JAI CV-S3300 CE (Edmund Optics, Barrington, NJ) and transmitted to a PC for recording. Videos were analyzed to quantify cells adhering per minute, whether rolling and firmly adherent. All of the following treatments occurred at 37^oC to preserve cell viability during treatment.

IL-8 inhibition. Fab fragments of anti-human IL-8 monoclonal antibody (R&D, 20ng/ml)) were added to both 15 Gy irradiated and unirradiated HAECs 30 minutes prior to adhesion assay to inhibit IL-8 produced by HAECs. Fab fragments of same host

species IgG were used to assess for nonspecific binding. Fab fragments were produced via digestion of monoclonal antibodies with ficin and cysteine via Fab fragment prep kit (Thermo Sci.) and verified by SDS-PAGE.

Media Transfer. Media were collected from 15 Gy irradiated and unirradiated HAECs 18 hours post-irradiation and stored at 4°C. Later collected media were added to unirradiated cells 24 hours prior to the adhesion assay. HAECs were stimulated with TNF-α 6 hours prior to adhesion assay as usual. 15 Gy irradiated HAECs act as a positive control and unirradiated HAECs as a negative control.

Effect of rhIL-8 on adhesion. Recombinant human IL-8 (10 ng/ml) was added to unirradiated cells 24 hours prior to adhesion assay. HAECs were stimulated with TNF-α 6 hours prior to adhesion assay as usual. 15 Gy irradiated HAECs act as a positive control and unirradiated HAECs as a negative control.

Effects of heparin on adhesion. Heparin (Sigma, 100 μg/ml) was added to both 15 Gy irradiated HAECs and control HAECs 30 minutes before assaying for adhesion. 15 Gy irradiated HAECs act as a positive control and unirradiated HAECs as a negative control.

Effects of heparitinase on adhesion. Heparitinase (Hep 3, Sigma, 100 mU/ml) was added to both 15 Gy irradiated HAECs and control HAECs two hours before assaying for adhesion. 15 Gy irradiated HAECs act as a positive control and unirradiated HAECs as a negative control.

RESULTS

Soluble proteins bound to cell surface GAGs are neccessary for adhesion of THP-1 cells to irradiated endothelial cells, but not unirradiated endothelial cells. Heparin is a highly sulfated GAG that binds chemokines and other GAG bound soluble factors with high affinity. When added to endothelial cultures, it inhibits chemokine signaling by competing away chemokines from cell surface GAG's. Heparin abrogated the radiation enhanced adhesion, but not the adhesion caused by TNF-α stimulation, indicating that soluble GAG bound factors are necessary for radiation enhanced adhesion.

Figure 1

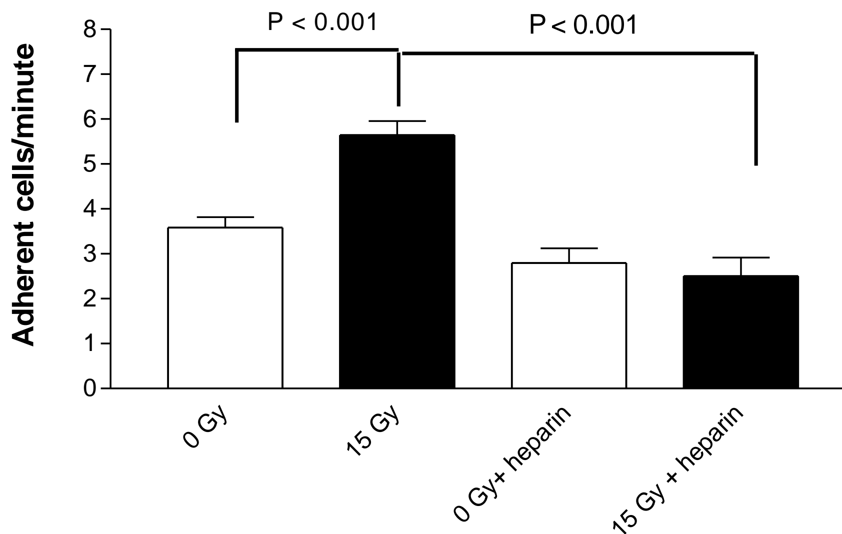


Figure 1. Heparin Addition to HAECs inhibits radiation enhanced adhesion but not TNF-α induced adhesion. *24 hours after irradiation of confluent HAEC monolayers adhesion of THP-1 cells under flow was measured with and without addition of Heparin. Heparin removes the chemokines from the endothelial cell surface heparan sulfate. Heparin significantly abrogated the radiation enhanced adhesion, but not adhesion induced by TNF-α alone (Mean± SEM, ANOVA with Bonferroni post-test).*

Endothelial cell surface Heparan Sulfate is required for adhesion of THP-1 cells to irradiated endothelial cells, but not unirradiated endothelial cells. Heparan sulfate is the most prominent GAG chain in the aortic endothelium, and binds chemokines with high affinity. Treating HAECs with heparitinase (Hep3) degrades surface heparan sulfate and inhibits radiation enhanced adhesion, indicating that heparan sulfate GAG chains are necessary for radiation enhanced adhesion. When the heparan sulfate is released from the HSPG core protein, all soluble factors bound to HS are also released. Hep3 treatment has no effect on adhesiveness of unirradiated HAECs. This indicates heparan sulfate and any HSPG bound factors are not required for TNF-α induced adhesion, but are necessary for radiation enhanced adhesion. This indicates that radiation enhanced adhesion requires HS bound chemokines when taken together with data from figure 1.

Figure 2

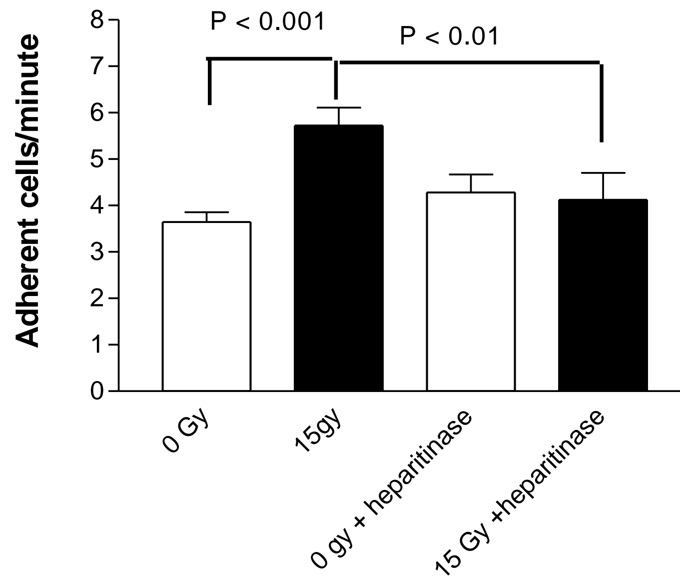


Figure 2. Enzymatic digestion of endothelial heparan sulfate by Hep3 inhibits radiation enhanced adhesion, but not TNF-α induced adhesion. *24 hours after irradiation of confluent HAEC monolayers adhesion of THP-1 cells under flow was measured with and without addition of Hep3, a heparan sulfate degrading enzyme. Radiation enhanced adhesion was significantly inhibited by removing heparan sulfate and any factors bound to it from the cell surface. Hep3 treatment had no effect on adhesion induced by TNF-α alone (Mean± SEM, ANOVA with Bonferroni post-test).*

A soluble factor(s) from irradiated HAECs enhances adhesion of THP-1 cells to unirradiated endothelial cells.

Media from 15 Gy irradiated cells 18 hours after irradiation and then added to unirradiated HAECs 24 hours prior to assaying endothelial adhesion (figure 3). This treatment with endothelial media from irradiated cells enhanced adhesion similarly to the irradiated endothelial cells. This indicates that soluble factors that are secreted by endothelial cells in response to radiation are an important factor in radiation enhanced adhesion.

Figure 3

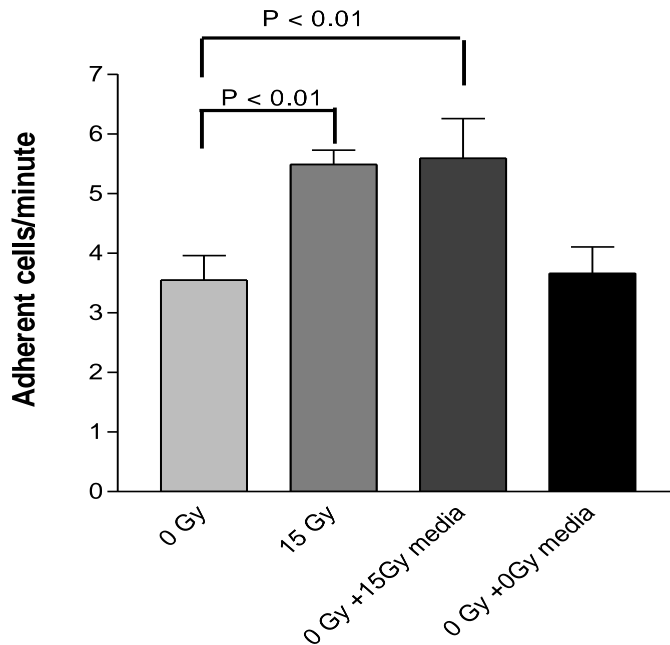


Figure 3. Addition of media from 15 Gy irradiated cells (without TNF-α) to unirradiated cells 24 hours prior to adhesion is sufficient for enhanced adhesion. *24 hours after irradiation of confluent HAEC monolayers, adhesion of THP-1 cells under flow was measured. Media from either 15 Gy or non-irradiated HAECs was collected 18 hours after radiation treatment and was then added to unirradiated HAECs 24 hours prior to adhesion assay. (Mean± SEM, ANOVA with Bonferroni post-test).*

IL-8 is the only chemokine showing significant and substantial increase in secretion 24 hours post radiation treatment. Media was collected from HAECs 24 hours after irradiation from both irradiated and unirradiated control cells and subjected to luminex assay to determine the concentration of multiple chemokines secreted by endothelial cells. Cells were pre-treated with or without TNF-α. IL-8 increased almost 5 fold after 15 Gy radiation as compared to sham irradiated control cells (Table 1). Whereas increases in MCP-3 secretion following radiation were statistically significant, the concentration of MCP-3 measured in all the samples was very low (<50 pg/ml) with respect to several of the other chemokines that were measured. Normally the concentration at which MCP-3 has chemotactic activity in an endothelial setting is at least 10 ng/ml (Ali et al. J. Immun. 2007). TNF-α stimulation of unirradiated cells did cause secretion of several chemokines. Radiation and TNF-α combined did not increase secretion of any of the chemokines tested. Several of the chemokines measured were not shown to be secreted by endothelial cells under any of the conditions.

Table 1

Mean Chemokine Concentration ± SEM (pg/ml)				
	0Gy	**0Gy+TNF-α**	**15 Gy**	**15 Gy + TNF-α**
IL-8	**1449 ± 131.8**	9694 ± 306.6	**7142 ± 1145**	10638 ± 1215
Gro-α	5871 ± 626.1	10932 ± 1266	6320 ± 81.41	7723 ± 679.3
CXCL9	13.25 ± 0.453	146.9 ± 43.59	18.23 ± 3.840	40.85 ± 14.53
MCP-3	6.912 ± 1.910	55.68 ± 8.947	38.64 ± 5.993	52.24 ± 9.658
MDC	8.898 ± 0.493	8.898 ± 0.493	10.52 ± 0.387	9.008 ± 0.595
CXCL10	22.17 ± 2.637	6792 ± 1559	48.32 ± 5.159	2890 ± 914.0
MCP-1	6287 ± 292.3	8768 ± 733.0	7674 ± 730.7	8187 ± 322.2
CX3CL1	10.2 ± 1.079	390.2 ± 79.17	18.1 ± 1.089	378.1 ± 91.76
CCL5	4.23 ± 1.136	203.3 ± 10.73	16.16 ± 5.305	145.7 ± 36.54
Not Detected:	CCL19 TARC MCP-4 SDF-1α Eotaxin MIP-1β MIP-1a			

Summary of changes		
	0 Gy vs. 15 Gy	**0Gy + TNF-α vs. 15 Gy+TNF-α**
IL-8	**↑ p<0.001**	↔
Gro-α	↔	↓P < 0.01
CXCL9	↔	↓P < 0.01
MCP-3	↑ p<0.01	↔
MDC	↔	↔
CXCL10	↔	↓P < 0.05
MCP-1	↔	↔
CX3CL1	↔	↔
CCL5	↔	↔

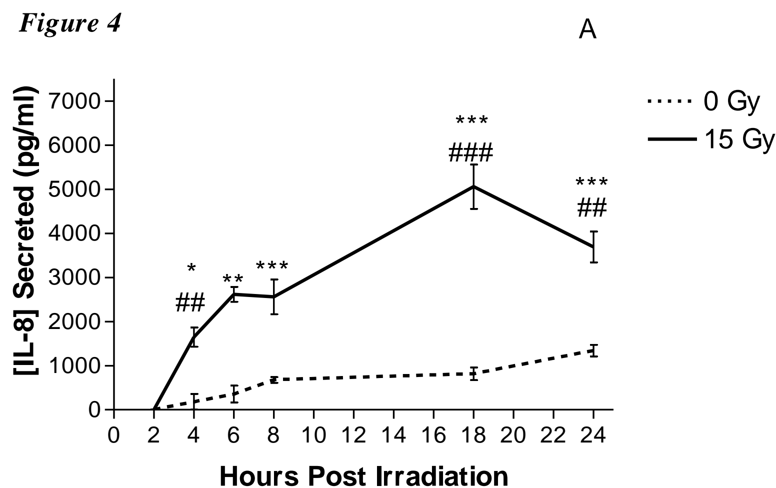
Table 1. Effects of radiation on secretion of multiple chemokines determined by Luminex assay. *15 Gy IR increased levels of IL-8 and MCP-3 in supernatant at 24 hours post IR without TNF-α present (MCP-3 levels were much lower than IL-8 however). (Mean± SEM, ANOVA with Bonferroni post-test).*

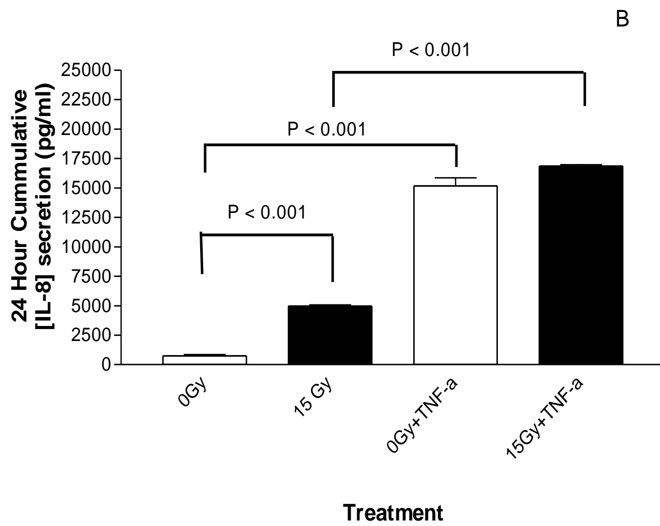
Radiation increases IL-8 secretion in a time dependent fashion indicating an acute response.

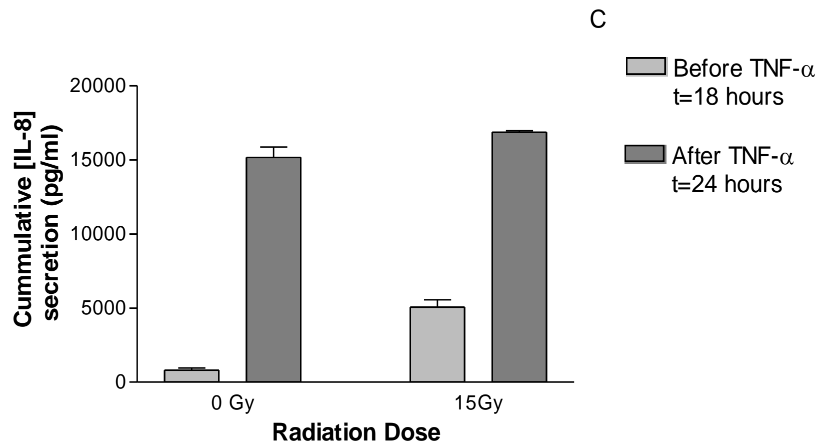
To confirm that radiation can induce IL-8 secretion, was an acute or chronic response, we measured cumulative IL-8 secretion over time from HAEC media collected between 2-24 hours post irradiation from both irradiated and unirradiated cells and analyzed by ELISA (Figure 4A). At two hours post-irradiation, no IL-8 was secreted. At four hours post irradiation IL-8 secretion was increased in media from 15 Gy irradiated cells relative to both the two hour time point and when compared to unirradiated cells. At six and eight hours post-irradiation, IL-8 secretion in 15 Gy irradiated cells was also increased compared to the unirradiated cells. At 18 hours post-irradiation the media from 15 Gy cells shows IL-8 secretion peaks at this time and is significantly increased from both the 15 Gy irradiated cells both from the eight hour time point and from the media collected from 18 hour unirradiated cells. At 24 hours the IL-8 secreted by the irradiated cells is also very high, though not at as much as the 18 hour time point, and is significantly increased from the small levels of IL-8 secreted by unirradiated cells over 24 hours. This pattern of IL-8 secretion is consistent with an acute response to X-ray irradiation, particularly due to the IL-8 secretion peaking at 18 hours and a lack of increase at 24 hours post irradiation. The fact that IL-8 is secreted in response to radiation prior to the time the cells would be treated with TNF-α indicates that the opportunity exists for IL-8 to bind cell surface GAG chains prior to the secretion of chemokines in response to TNF-α. To determine the relation of IL-8 secreted over time to the IL-8 secreted in the adhesion assay conditions, and confirm the results of the luminex assay, the media was collected from irradiated and unirradiated cells treated with and without TNF-α after 24

hours and analyzed by ELISA (Figure 4B). The unirradiated cells and 15 Gy irradiated cells treated with TNF-α contained similar concentrations of IL-8. The 15 Gy irradiated cells without TNF-α contained significantly more IL-8 than unstimulated unirradiated cells. The data in Figure 4A indicates that 5000 pg/ml of IL-8 was secreted in response to radiation in the time prior to TNF-α stimulation, which is 30% of the total IL-8 secreted in response to both TNF-α and radiation (Figure 4C). This indicates that irradiated cells actually produce less IL-8 in response to TNF-α than unirradiated cells do or that irradiated cells sequester IL-8 more readily than unirradiated cells so that less IL-8 is free in the media.

Figure 4




B
P < 0.001
P < 0.001
P < 0.001
24 Hour Cummulative [IL-8] secretion (pg/ml)
25000
22500
20000
17500
15000
12500
10000
7500
5000
2500
0
0Gy
15 Gy
0Gy+TNF-a
15Gy+TNF-a
Treatment


C
Before TNF-α
t=18 hours
After TNF-α
t=24 hours
Cummulative [IL-8] secretion (pg/ml)
20000
15000
10000
5000
0
0 Gy
15Gy
Radiation Dose

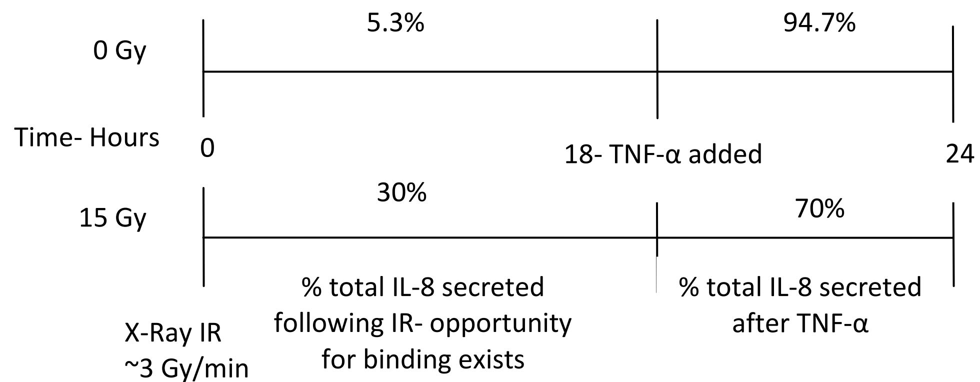

0 Gy
5.3%
94.7%
Time- Hours
0
18- TNF-α added
24
15 Gy
30%
70%
% total IL-8 secreted following IR- opportunity for binding exists
% total IL-8 secreted after TNF-α
X-Ray IR
~3 Gy/min

Figure 4. IL-8 is secreted by HAECs in a time dependent manner consistent with an acute response to radiation. ***A:*** *IL-8 secretion was measured via ELISA from media collected from cells 2, 4, 6, 8, 18, and 24 hours post irradiation. Similarly treated unirradiated cells served as a control to determine baseline IL-8 secretion. Secretion of Il-8 began four hours post irradiation and peaked at 18 hours post irradiation.0 Gy vs. 15 Gy: p<0.05=*, p<0.01=**, p<0.001=*** , 15 Gy vs previous15 Gy time point: p<0.05=#, p<0.01=##, p<0.001=### (Mean± SEM, ANOVA with Bonferroni post-test)* ***B:*** *IL-8 secretion was measured via ELISA from media collected from cells 24 hours after 15 Gy irradiation with or without TNF-α for the last six hours. Radiation significantly increased IL-8 secretion compared to unirradiated cells, while no change was observed between secreted IL-8 from TNF-α stimulated unirradiated and 15 Gy irradiated cells. TNF-α alone caused significant secretion of IL-8 from unirrradiated cells. Irradiated HAECs also showed significant increase in secretion following TNF-α stimulation (Mean± SEM, ANOVA with Bonferroni post-test).* ***C:*** *18 Hour time points from panel A is shown together with 24 hour time points with TNF- α from panel B. This shows that 15 Gy irradiated cells secrete more IL-8 in response to radiation prior to TNF- α and secrete less IL-8 in response to TNF- α compared to unirradiated cells. The diagram below panel C shows that data converted to % of total IL-8 secreted before and after TNF-α is added.*

Inhibiting endothelial IL-8 inhibits radiation enhanced adhesion. To determine if IL-8 was necessary for adhesion we treated HAECs with anti-IL-8 Fab for 30 minutes prior to adhesion assay. IL-8 Fab fragments inhibited radiation enhanced adhesion, and had no effect on adhesiveness of unirradiated cells. IgG fab fragments had no significant effect on either TNF-α induced or radiation enhanced adhesion (figure 5). This shows that IL-8 is not necessary for baseline, TNF-α induced adhesiveness, but is required for the radiation enhanced component of adhesiveness.

Figure 5

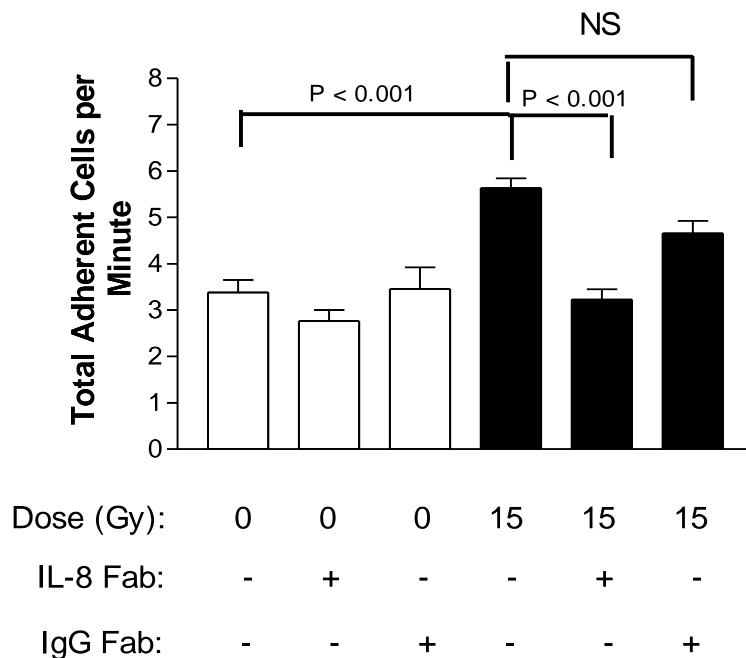


Figure 5. Anti- lL-8 Fab fragments inhibit radiation enhanced adhesion, but not TNF-α induced adhesion. *24 hours after irradiation of confluent HAEC monolayers, adhesion of THP-1 cells under flow was measured with and without addition of anti-IL-8 Fab fragments or control IgG Fab fragments 30 minutes prior to adhesion assay. Anti-IL-8 Fab fragments abolished the increase in adhesion due to radiation but had no effect on baseline adhesion induced by TNF-α alone. Control IgG fab fragments had no significant effect on any adhesion (Mean± SEM, ANOVA with Bonferroni post-test).*

Recombinant human IL-8 enhances adhesion of THP-1 cells to unirradiated endothelial cells.

In a similar experiment, recombinant human IL-8 was added to unirradiated HAECs 24 hours prior to assaying endothelial adhesion (figure 6). 10 ng/ml of rhIL-8 added to endothelial cells was added to endothelial cells 24 hours prior to adhesion assay to determine if IL-8 added prior to TNF-α treatment was able to enhance endothelial adhesion. Treatment of HAECs with recombinant IL-8 enhanced endothelial adhesiveness to the same extent as radiation of endothelial cells enhanced adhesion.

Figure 6

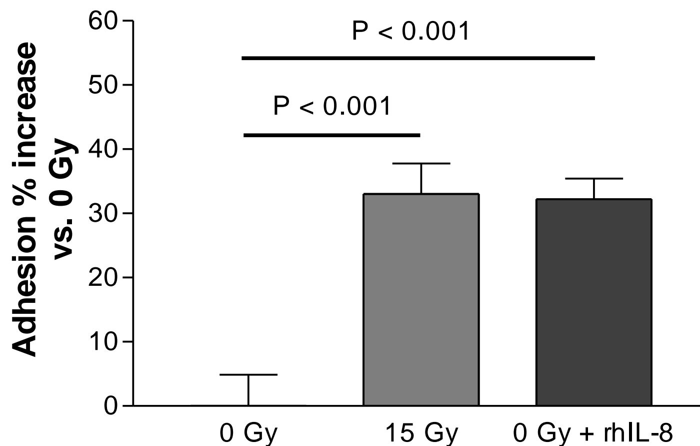


Figure 6. Addition of rhIL-8 to unirradiated cells 24 hours prior to adhesion assay is sufficient for enhanced adhesion. *24 hours after irradiation of confluent HAEC monolayers adhesion of THP-1 cells under flow was measured. 10 ng/ml recombinant human IL-8 was added to unirradiated HAECs 24 hours prior to adhesion assay with normal TNF-α treatment. (Mean± SEM, ANOVA with Bonferroni post-test).*

Figure 7

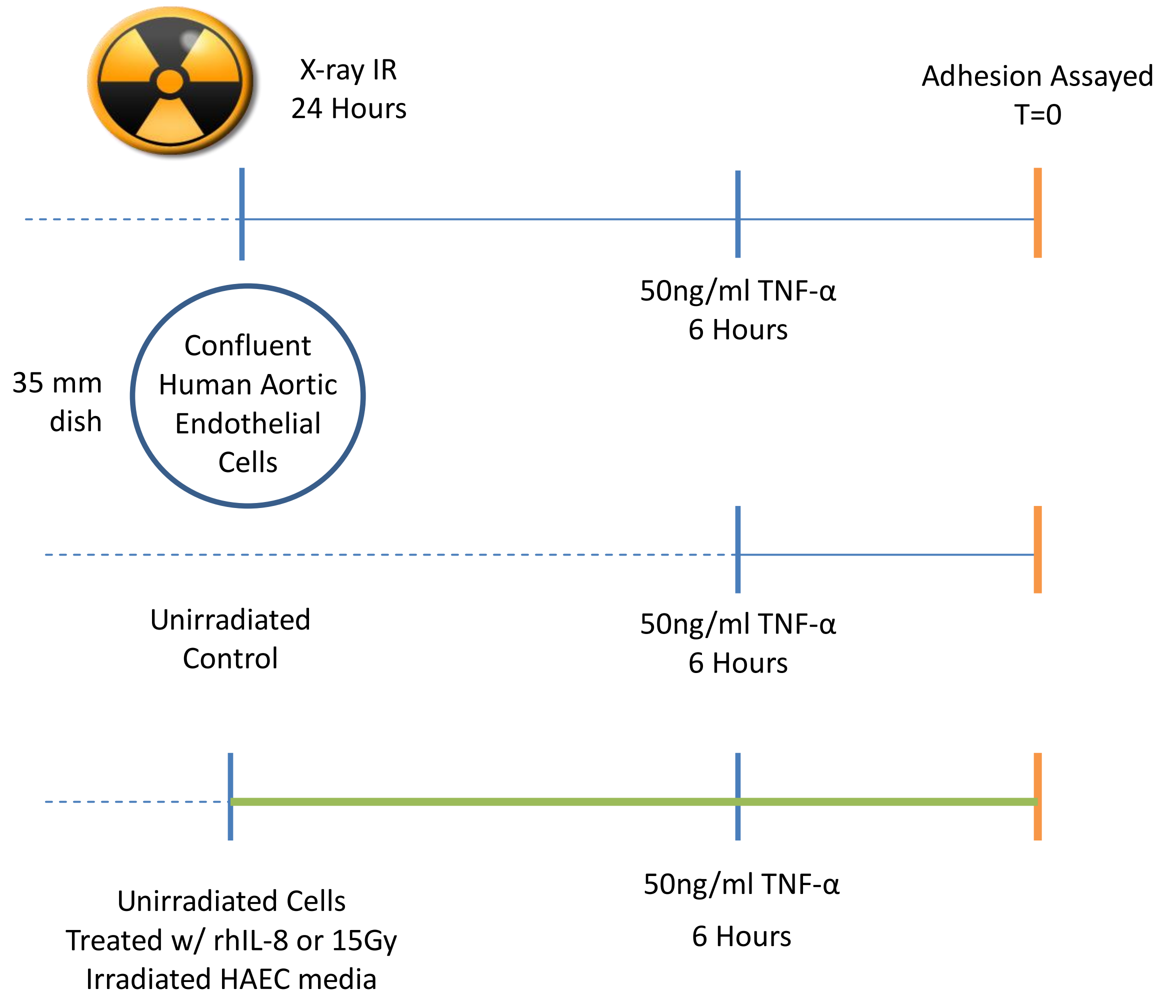


Figure 7. Diagram showing treatment timeline HAECs undergo prior to adhesion assay. *Confluent human aortic endothelial cells are irradiated 24 hours prior to adhesion assay. TNF-α treatment occurs 6 hours prior to adhesion assay (18 hours after radiation). The cells treated with rhIL-8 are treated 24 hours to the adhesion assay. The media from 15 Gy irradiated HAECs used to treat unirradiated HAECs is collected 18 hours after irradiation and added 24 hours before the adhesion assay.*

DISCUSSION

Previously much of the mechanism resulting in radiation enhanced endothelial adhesiveness was unknown. Integrin ligand expression is unchanged after radiation treatment and therefore integrin activation must be increased for radiation enhanced adhesion. It is known that the expression of the endothelial integrin ligands ICAM-1 and VCAM-1 is unchanged by radiation at the time adhesion was assayed (Khaled et al. Rad. Res. 2011). THP-1 cells were not exposed to radiation and therefore were responding to changes in molecules presented on the endothelial cell surface. Because our findings show that increased adhesion occurs after radiation and that this occurred in the absence of an increase in integrin ligands, this presents a previously undiscovered mechanism in that we show radiation enhanced adhesion is chemokine dependent and occurs when ligand expression is not increased (Khaled et al. Rad. Res. 2011). In this study we advance the previous findings by demonstrating that IL-8 is the driving force behind radiation enhanced adhesion. This agrees with previous findings showing the activation of chemokine receptors and subsequent activation of integrins is critical for radiation enhanced adhesion.

Chemokine activation of monocyte integrins via chemokine receptors is critical for endothelial adhesion and for the inflammatory cascade. Previous studies have shown that both select integrins and chemokine receptors play a role in radiation enhanced adhesion (Khaled et al. Rad. Res. 2011). Irradiation of cultured human aortic endothelial cells

results in a release of IL-8 and subsequent enhanced adhesion is mediated, at least in part, by IL-8. This response is an active response and not a result of radiation induced cell death. At the time adhesion was assayed apoptosis or necrosis was not increased as determined by annexin V-Propidium Iodide FACS, indicating that radiation did not compromise cell membrane stability (Khaled et al. Rad. Res. 2011). This is to be expected as endothelial cells grown to confluence will have a very slow rate of growth, if any, and normally irradiation has a strong negative impact on cell viability in cases where the cells are rapidly undergoing cell division, such as epithelial or cancer cells. This is also consistent with our previously published data showing that HAECs do not undergo apoptosis or necrosis in response to radiation (Khaled et al. Rad. Res. 2011). IL-8 was the only chemokine tested that was secreted in biologically meaningful quantities in response to radiation. Inhibition of endothelial IL-8 only inhibited the adhesion enhanced by radiation and not the basal adhesion that resulted from TNF-α stimulation. When treating unirradiated endothelial cells with rhIL-8 it enhanced adhesiveness of the endothelial cells under flow conditions. These data taken together demonstrate that IL-8 secreted by aortic endothelial cells in response to radiation is both necessary and sufficient for radiation enhanced adhesion.

Increased IL-8 secretion by endothelial cells in response to radiation is consistent with previous studies by other groups (Meeren et al. Mediators of Inflammation 1997, Blirando et al. Rad.Res. 2012). However it remained unknown whether or not IL-8 released in response to radiation had any functional consequence. The work presented by our group advances these findings by demonstrating a functional role of radiation induced IL-8 secretion is to enhance endothelial adhesiveness. We have furthered the

understanding of the effects of radiation on IL-8 secretion by showing that IL-8 is necessary and sufficient for radiation enhanced adhesion. Furthermore our data suggests that both IL-8 and endothelial heparan sulfate (HS) are responsible for enhancing endothelial adhesiveness in response to radiation.

IL-8 is a known inflammatory chemokine that mediates adhesion via presentation by endothelial proteoglycans. Because of its soluble nature, IL-8 presentation is necessary for it to trigger adhesion via chemokine receptors present on the surface of THP-1 cells. When chemokines bind surface GAG chains they allow haptotaxis, movement from an area of low chemokine concentration on the cell surface to an area of high concentration, of circulating cells when they contact the endothelium. When determining the time at which radiation induces IL-8 secretion we have shown that IL-8 peaks at 18 hours post 15 Gy irradiation, the time at which TNF-a treatment occurs, indicating an acute response by endothelial cells to radiation. At 24 hours post irradiation the concentration of IL-8 was still high relative to the earliest time points; however it was significantly lower than at 18 hours. This could result from a combination of concentration dependent internalization of IL-8 via CXCR1/CXCR2 and/or cells retaining more IL-8 on the surface via proteoglycans. It is also possible that because in response to radiation IL-8 is secreted prior to the TNF-α stimulation and therefore does not have to compete for surface binding with soluble factors that are secreted as a result of TNF-α stimulation. *In vivo* it is possible that a release of IL-8 into the vasculature following radiation exposure would lead to increased adhesion of those cells to the aortic endothelium. In our system removal of endothelial HS, the most prominent GAG species of the aortic endothelium, inhibited the enhanced adhesion in response to radiation. As researchers in the the field

develop a greater understanding of the interaction between chemokines and cell surface proteoglycans we will be able to better understand the effects of radiation on vascular inflammation.

Much of what is known about IL-8 has not specifically linked it to distinct diseases, although its receptors which are promiscuous have been linked to atherosclerosis in mice. Much of the previous work done has described IL-8 as a non-specific mediator of inflammation in multiple organ systems (Yu et al. IJBS. 2013, Bendre et al. Bone. 2002, 2003). Our findings advance what is known about IL-8 related pathological mechanisms by showing that it is capable of mediating adhesion enhanced by radiation. We also showed that treating unirradiated HAECs with IL-8 prior to TNF-α stimulation was enough by itself to increase adhesion compared to cells only treated with TNF-α (figure 6). Increased adhesion is a critical early event in atherosclerosis. IL-8 is an important mediator of radiation enhanced endothelial adhesiveness, a key cause of inflammation in the vasculature that contributes to atherosclerosis.

FUTURE PERSPECTIVES

We show that radiation increases the secretion of IL-8. In human endothelial cells whether increased expression or increased secretion of IL-8 is critical for this effect is unknown. Others have shown previously that chemokines tend to be stored in cytoplasmic bodies with other chemokines (Rot et al. J Leukoc Biol. 1996). Therefore, increased expression of IL-8 would be more likely than an increase in rate of secretion due to our findings showing no increase in secretion of chemokines other than IL-8 (Table 1). To determine if radiation increases IL-8 expression measuring mRNA would Elucidating the mechanism by which radiation causes an increase in IL-8 expression would certainly provide additional insight and possible targets for therapeutic countermeasures.

These findings can provide a basis for future in vivo studies to determine if a similar IL-8 dependent mechanism plays a role in animal models that have shown accelerated atherosclerosis. We can assess if increased levels of IL-8 are found in circulation following radiation exposure. Furthermore, in these animal studies we could test if IL-8 receptor inhibitors are able to attenuate atherosclerosis accelerated by radiation. If success is shown in in vivo studies, IL-8 and its receptors can be considered for therapeutic targets for individuals who are exposed to radiation such as cancer patients or astronauts on deep space missions.

While these findings further our understanding of the way radiation enhances adhesion via chemokines, the underlying mechanism behind how radiation increases IL-8 release from endothelial cells is still unknown. Is the increase in IL-8 secretion in response to radiation due to increased expression of IL-8 by endothelial cells, and if so what are the molecular targets of radiation that result in increases in IL-8 secretion? It could be possible that oxidative stress induced by radiation plays a part in the chemokine/IL-8 response. Another explanation might be that radiation changes the way endothelial cells traffic pro-inflammatory signaling proteins such as IL-8.

In our *in vitro* system our data showed that we can detect IL-8 secretion from human endothelial cells in response to radiation as soon as 4 hours post radiation exposure. This suggests that it could be possible to use IL-8 in serum diagnostically to determine if individuals are exposed to radiation could suffer from inflammation associated with radiation soon after exposure. If IL-8 acts to trigger downstream inflammatory responses, such as increasing adhesiveness of nearby endothelial cells, there could be even more severe consequences such as formation of additional lesions. Because IL-8 can undergo proteolysis to increase the chemotactic activity environments that contain increases in the pro-inflammatory proteases thrombin and plasmin, radiation being one example, IL-8 may in fact have more potent activity in individuals exposed to radiation. It is possible that the ability of IL-8 to bind HS is also enhanced by radiation via proteases and the increase in HSPG bound IL-8 is a potential factor in radiation enhanced adhesion.

LIST OF REFERENCES

Ali, S., Robertson, H., Wain, J. H., Isaacs, J. D., Malik, G., & Kirby, J. A. (2005). A non-glycosaminoglycan-binding variant of CC chemokine ligand 7 (monocyte chemoattractant protein-3) antagonizes chemokine-mediated inflammation. *J.Immunol., 175,* 1257-1266.

Axelsson, J., Xu, D., Kang, B. N., Nussbacher, J. K., Handel, T. M., Ley, K. et al. (2012). Inactivation of heparan sulfate 2-O-sulfotransferase accentuates neutrophil infiltration during acute inflammation in mice. *Blood, 120,* 1742-1751.

Bao, Z., Ye, Q., Gong, W., Xiang, Y., & Wan, H. (2010). Humanized monoclonal antibody against the chemokine CXCL-8 (IL-8) effectively prevents acute lung injury. *Int.Immunopharmacol., 10,* 259-263.

Bendre, M. S., Gaddy-Kurten, D., Mon-Foote, T., Akel, N. S., Skinner, R. A., Nicholas, R. W. et al. (2002). Expression of interleukin 8 and not parathyroid hormone-related protein by human breast cancer cells correlates with bone metastasis in vivo. *Cancer Res., 62,* 5571-5579.

Bendre, M. S., Montague, D. C., Peery, T., Akel, N. S., Gaddy, D., & Suva, L. J. (2003). Interleukin-8 stimulation of osteoclastogenesis and bone resorption is a mechanism for the increased osteolysis of metastatic bone disease. *Bone, 33,* 28-37.

Berliner, J. A., Navab, M., Fogelman, A. M., Frank, J. S., Demer, L. L., Edwards, P. A. et al. (1995). Atherosclerosis: basic mechanisms. Oxidation, inflammation, and genetics. *Circulation, 91,* 2488-2496.

Blirando, K., Hneino, M., Martelly, I., Benderitter, M., Milliat, F., & Francois, A. (2012). Mast cells and ionizing radiation induce a synergistic expression of inflammatory genes in endothelial cells by a mechanism involving p38alpha MAP kinase and (p65) NF-kappaB activation. *Radiat.Res., 178,* 556-567.

Boisvert, W. A., Santiago, R., Curtiss, L. K., & Terkeltaub, R. A. (1998). A leukocyte homologue of the IL-8 receptor CXCR-2 mediates the accumulation of

macrophages in atherosclerotic lesions of LDL receptor-deficient mice. *J.Clin.Invest, 101,* 353-363.

Burns, D. L. (1988). Subunit structure and enzymic activity of pertussis toxin. *Microbiol.Sci., 5,* 285-287.

Carveth, H. J., Bohnsack, J. F., McIntyre, T. M., Baggiolini, M., Prescott, S. M., & Zimmerman, G. A. (1989). Neutrophil activating factor (NAF) induces polymorphonuclear leukocyte adherence to endothelial cells and to subendothelial matrix proteins. *Biochem.Biophys.Res.Commun., 162,* 387-393.

Cheng, S. W., Wu, L. L., Ting, A. C., Lau, H., Lam, L. K., & Wei, W. I. (1999). Irradiation-induced extracranial carotid stenosis in patients with head and neck malignancies. *Am.J.Surg., 178,* 323-328.

Collins, R. G., Velji, R., Guevara, N. V., Hicks, M. J., Chan, L., & Beaudet, A. L. (2000). P-Selectin or intercellular adhesion molecule (ICAM)-1 deficiency substantially protects against atherosclerosis in apolipoprotein E-deficient mice. *J.Exp.Med., 191,* 189-194.

Hauptmann, M., Mohan, A. K., Doody, M. M., Linet, M. S., & Mabuchi, K. (2003). Mortality from diseases of the circulatory system in radiologic technologists in the United States. *Am.J.Epidemiol., 157,* 239-248.

Heit, B., Colarusso, P., & Kubes, P. (2005). Fundamentally different roles for LFA-1, Mac-1 and alpha4-integrin in neutrophil chemotaxis. *J.Cell Sci., 118,* 5205-5220.

Hneino, M., Blirando, K., Buard, V., Tarlet, G., Benderitter, M., Hoodless, P. et al. (2012). The TG-interacting factor TGIF1 regulates stress-induced proinflammatory phenotype of endothelial cells. *J.Biol.Chem., 287,* 38913-38921.

Khaled, S., Gupta, K. B., & Kucik, D. F. (2012). Ionizing radiation increases adhesiveness of human aortic endothelial cells via a chemokine-dependent mechanism. *Radiat.Res., 177,* 594-601.

Langer, H. F. & Chavakis, T. (2009). Leukocyte-endothelial interactions in inflammation. *J.Cell Mol.Med., 13,* 1211-1220.

Leong, S. R., Lowman, H. B., Liu, J., Shire, S., Deforge, L. E., Gillece-Castro, B. L. et al. (1997). IL-8 single-chain homodimers and heterodimers: interactions with chemokine receptors CXCR1, CXCR2, and DARC. *Protein Sci., 6,* 609-617.

Liao, F., Andalibi, A., deBeer, F. C., Fogelman, A. M., & Lusis, A. J. (1993). Genetic control of inflammatory gene induction and NF-kappa B-like transcription factor activation in response to an atherogenic diet in mice. *J.Clin.Invest, 91,* 2572-2579.

Liao, F., Andalibi, A., Qiao, J. H., Allayee, H., Fogelman, A. M., & Lusis, A. J. (1994). Genetic evidence for a common pathway mediating oxidative stress, inflammatory gene induction, and aortic fatty streak formation in mice. *J.Clin.Invest, 94,* 877-884.

Libby, P., Ridker, P. M., & Hansson, G. K. (2009). Inflammation in atherosclerosis: from pathophysiology to practice. *J.Am.Coll.Cardiol., 54,* 2129-2138.

Lomakina, E. B. & Waugh, R. E. (2010). Signaling and Dynamics of Activation of LFA-1 and Mac-1 by Immobilized IL-8. *Cell Mol.Bioeng., 3,* 106-116.

Matesanz, N., Jewhurst, V., Trimble, E. R., McGinty, A., Owens, D., Tomkin, G. H. et al. (2012). Linoleic acid increases monocyte chemotaxis and adhesion to human aortic endothelial cells through protein kinase C- and cyclooxygenase-2-dependent mechanisms. *J.Nutr.Biochem., 23,* 685-690.

Meeren, A. V., Bertho, J. M., Vandamme, M., & Gaugler, M. H. (1997). Ionizing radiation enhances IL-6 and IL-8 production by human endothelial cells. *Mediators.Inflamm., 6,* 185-193.

Mortier, A., Berghmans, N., Ronsse, I., Grauwen, K., Stegen, S., Van, D. J. et al. (2011). Biological activity of CXCL8 forms generated by alternative cleavage of the signal peptide or by aminopeptidase-mediated truncation. *PLoS.One., 6,* e23913.

Proudfoot, A. E. (2006). The biological relevance of chemokine-proteoglycan interactions. *Biochem.Soc.Trans., 34,* 422-426.

Rot, A., Hub, E., Middleton, J., Pons, F., Rabeck, C., Thierer, K. et al. (1996). Some aspects of IL-8 pathophysiology. III: Chemokine interaction with endothelial cells. *J.Leukoc.Biol., 59,* 39-44.

Schwartz, D., Andalibi, A., Chaverri-Almada, L., Berliner, J. A., Kirchgessner, T., Fang, Z. T. et al. (1994). Role of the GRO family of chemokines in monocyte adhesion to MM-LDL-stimulated endothelium. *J.Clin.Invest, 94,* 1968-1973.

Shimizu, Y., Kodama, K., Nishi, N., Kasagi, F., Suyama, A., Soda, M. et al. (2010). Radiation exposure and circulatory disease risk: Hiroshima and Nagasaki atomic bomb survivor data, 1950-2003. *BMJ, 340,* b5349.

Sima, A. V., Stancu, C. S., & Simionescu, M. (2009). Vascular endothelium in atherosclerosis. *Cell Tissue Res., 335,* 191-203.

Simionescu, N., Vasile, E., Lupu, F., Popescu, G., & Simionescu, M. (1986). Prelesional events in atherogenesis. Accumulation of extracellular cholesterol-rich liposomes in the arterial intima and cardiac valves of the hyperlipidemic rabbit. *Am.J.Pathol., 123,* 109-125.

Springer, T. A. (1994). Traffic signals for lymphocyte recirculation and leukocyte emigration: the multistep paradigm. *Cell, 76,* 301-314.

Wang, L., Fuster, M., Sriramarao, P., & Esko, J. D. (2005). Endothelial heparan sulfate deficiency impairs L-selectin- and chemokine-mediated neutrophil trafficking during inflammatory responses. *Nat.Immunol., 6,* 902-910.

Witztum, J. L. (1994). The oxidation hypothesis of atherosclerosis. *Lancet, 344,* 793-795.

Yang, C. R., Hsieh, S. L., Ho, F. M., & Lin, W. W. (2005). Decoy receptor 3 increases monocyte adhesion to endothelial cells via NF-kappa B-dependent up-regulation of intercellular adhesion molecule-1, VCAM-1, and IL-8 expression. *J.Immunol., 174,* 1647-1656.

Yu, H., Huang, X., Ma, Y., Gao, M., Wang, O., Gao, T. et al. (2013). Interleukin-8 regulates endothelial permeability by down-regulation of tight junction but not dependent on integrins induced focal adhesions. *Int.J.Biol.Sci., 9,* 966-979.