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Analysis of molecular switch between leukocyte and substrate adhesion in bone marrow endothelial cells

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ABSTRACT

Aim: Endothelial cell damage is critical to understand since its presence in the entire body makes the damage widespread instead of being localized. Being a major component of stem cell niche in bone marrow, deems it essential to gain knowledge of the damage to endothelium associated with bone marrow. Since radiation exposure has become common to numerous therapeutic modalities, its effects on bone marrow and its endothelial cells are crucial to understand.

Material & methods: Microarray analysis was performed on irradiated human bone marrow endothelial cells (hBMECs) with and without prior treatment with radioprotectant amifostine to assess the effects of radiation on signalling pathways and the subsequent changes in pathways when treated with radioprotectant prior to radiation exposure.

Key findings: It was seen that adhesion pathways that were usually inactivated under normal circumstances were stimulated post radiation. However, where in the case of radiation exposure, these adhesion pathways included leukocyte adhesion and migration; in the case of radioprotected conditions the pathways revolve around cell-substrate adhesion and cell spreading. Genes like ROCK1, FLNA, RAC1, PRKCZ and MAP3K8 were seen to regulate the molecular switch between leukocyte-cell adhesion to cell-substrate adhesion.

Significance: Our study demonstrated that irradiated endothelium supports leukocyte adhesion and migration but shifts to substrate adhesion dependent cell spreading under radioprotected conditions in order to repair the monolayer damage from the radiation. The genes responsible for the shift were identified and can be employed to manipulate cell adhesion characteristics for the treatment of diseases caused by radiation or inflammation.

1. Introduction

Radiation injury is characterized by loss of cell count, tissue damage, vascular lesion and coagulation disorders [1–4]. Vascular lesion is described as the loss of vessel wall integrity due to apoptosis of endothelial cells lining the vasculature causing increased vessel permeability and consequential tissue edema. In addition to being a selective barrier, endothelium also maintains vascular homeostasis by modifying its surface properties. It has been observed that under normal circumstances endothelium maintains a non-adhesive surface which switches to adhesive state in response to a myriad of inflammatory cues. For instance, any injury triggers the cells to promote platelet adhesion causing fibrin deposition and thrombosis subsequently leading to vascular occlusion and tissue ischemia [5–8] whereas adhesion of leukocytes to vessel wall followed by their migration, tissue infiltration, tissue damage by releasing reactive oxygen species [9] is a common inflammatory phenomenon. Being spread throughout the body, the effects of these vascular damages are not confined locally instead are global in nature damaging various organs, resulting in multiple system failure.

Since bone marrow inhabited by dividing hematopoietic stem cells holds the capacity to reconstitute the declined blood cell count induced by radiation, it becomes critical to study the endothelial cells associated with bone marrow and the damage inflicted on them by radiation as well as the changes in endothelial behaviour when shielded from radiation exposure via prior treatment with radioprotectant specifically in terms of cell adhesion. Although it has been established that radiation induces the increased expression of adhesion molecules on endothelial cells like VCAM1, ICAM1, PECAM1, E-selectin, CD44, VE-Cadherin, PSGL, etc. [10–12], no study has yet been reported that explains the changes in signalling pathways when endothelial cells were treated with radioprotectant before irradiation. It can only be hypothesised that

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upregulated adhesion molecules might return to their baseline levels in radioprotected conditions.

Hence we conducted microarray analysis of human bone marrow endothelial cells (hBMECs) and explored the pathways activated due to radiation exposure (RAD) and the deviations from them when administered with a radioprotectant preceding radiation (R + A).

It was observed that pathways relevant to adhesion remained active in R + A treatment but instead changed their nature. The pathways switched from leukocyte-cell adhesion in RAD samples to substrate-cell adhesion in R + A samples. Further analysis indicated that ROCK1, FLNA, RAC1, PRKCZ and MAP3K8 genes were responsible to regulate this switch, where RAC1 and ROCK1 attributed to cytoskeleton remodelling, FLNA linked cytoskeleton to membrane and MAP3K8 and PRKCZ were seen to regulate cell-cell adhesion.

2. Material & Methods

2.1. Reagents

Endothelial Cell Growth Medium (ECGM) and flask coated with extracellular matrix (ECM) were purchased from Celprogen (California, USA). Fetal bovine serum (FBS) and penicillin-streptomycin-amphotericin B solution were obtained from Gibco, Thermo Fisher Scientific. RNA*later* stabilizing solution and TRIzol solution were procured from Invitrogen, Thermo Fisher Scientific. Trypsin was procured from Himedia Pvt. Ltd. and ethylenediaminetetraacetic acid (EDTA) and amifostine trihydrate were procured from Sigma Aldrich. Sodium chloride (NaCl), potassium chloride (KCl), disodium hydrogen phosphate (Na_2HPO_4) and potassium dihydrogen phosphate (KH_2PO_4) were obtained from Merck. Phosphate buffered saline was prepared with 137mM NaCl, 2.7mM KCl, 10mM Na_2HPO_4, 1.8mM KH_2PO_4.

2.2. Cell culture

Primary endothelial cells derived from adult human bone marrow (hBMECs) were procured from Celprogen (Torrance, USA) at passage 2 and were cultured in ECGM supplemented with 10% heat-inactivated fetal bovine serum (FBS), 100units/ml penicillin, 100μg/ml streptomycin and 250 ng/ml of amphotericin B. Cells were plated in flasks precoated with extra-cellular matrix (ECM) and incubated at 37 °C in 5% CO_2 humidified incubator. Media was replaced with fresh media in every two days. At 60–70% confluency, cells were washed with phosphate buffered saline (PBS) and treated with 0.25% trypsin-EDTA solution for 2–5 min. Fresh media was added to inactivate trypsin and cells were centrifuged at 200 × *g*, 5 min. The pellet obtained was resuspended in media and cells were plated.

2.3. RNA extraction

Amifostine trihydrate was dissolved in ECGM and filter sterilized. hBMECs taken at passage 5 were treated with amifostine solution at 6mM concentration (Supplementary Fig. 1 and Fig 2) for 30 min [13]. Cells were gamma irradiated at 6Gy by Cobalt-60 Bhabhatron II irradiator. Four groups were taken:

Group 1: Nonirradiated cells without amifostine treatment (CON)
Group 2: Cells treated with 6mM amifostine for 30 min (AMF)
Group 3: Cells irradiated at 6Gy (RAD)
Group 4: Cells treated with 6mM amifostine for 30 min and then irradiated at 6Gy (R + A)

Media was replaced with fresh media in groups 2, 3 and 4 after treatment. Group 1 containing nonirradiated cells without amifostine treatment were taken as control (CON). Cells were incubated for 24 hrs at 37 °C in 5% CO_2 humidified incubator followed by trypsinization. Cells were washed with PBS and pelleted at 200 × *g*, 5 min. The pellet was preserved in RNA*later* stabilizing solution followed by snap-freeze in liquid nitrogen and shipped to Genotypic Technology Pvt. Ltd. for further processing. Pellet was then thawed and RNA was extracted by TRIzol method. Concentration and purity of RNA were evaluated by Nanodrop spectrophotometer (Thermo Scientific) and integrity were analysed by Bioanalyzer (Agilent).

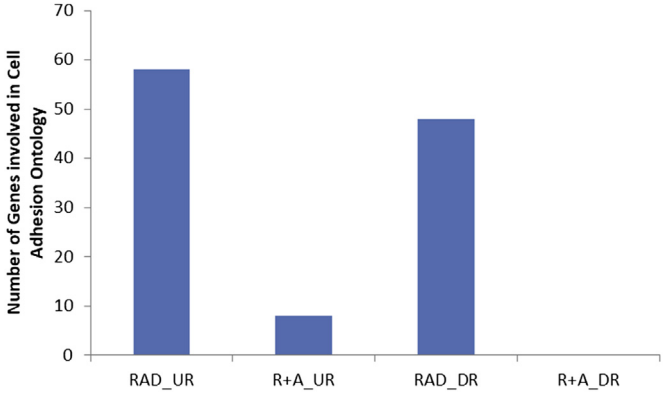

Fig. 1. Number of upregulated (UR) and downregulated (DR) genes involved in Gene Ontologies for Cell Adhesion in irradiated hBMECs (RAD) and amifostine pretreated irradiated hBMECs (R + A) among the differentially expressed genes with -2.0 ≥ FC ≥ 2.0 and p-value ≤ 0.05 as determined by DAVID online software.

2.4. Microarray analysis

cRNA was labelled with Cy3 by using Agilent Quick Amp labelling kit following manufacturer's instructions. Labelled cRNA was cleaned using Qiagen Rneasy columns. Dye corporation and cRNA yield were checked by Nanodrop ND-1000. The labelled cRNA was fragmented at 60 °C and hybridized on to Agilent Human Gene Expression microarray 8 × 60k array designed by Genotypic Technology Pvt. Ltd. using Agilent's In Situ Hybridization Kit. Scanning was done using Agilent microarray scanner and raw data was analysed by using GeneSpring GX software (Agilent). The gProcessed signal (dye normalized background subtracted signal intensity) was log base 2 transformed and then for each of the array the 75th percentile value was calculated separately. In each sample the log transformed intensity values for each probe is subtracted by the calculated 75th percentile value of the respective array and expression values were obtained. Fold change (FC) of the expression values was calculated with respect to CON group and its Geomean was determined.

Accession Number: Data has been uploaded in NCBI GEO database with accession number GSE108722 to be published soon.

2.5. Gene ontology and enrichment analysis

Geomean values of the fold change (FC) with respect to control and p-value across biological triplicate values for samples were analysed and differentially expressed genes (DEGs) were selected on the basis of -2.0 ≥ FC ≥ 2 and p-value ≤ 0.05. DEGs for each condition were examined by DAVID (Database for Annotation, Visualization and Integrated Discovery) version 6.8 for annotation and gene ontology enrichment analysis [14,15] with EASE (Expression Analysis Systematic Explorer) score of 0.1 [16].

2.6. Protein-protein interaction (PPI)

Selected gene ontologies from DAVID were further assessed by STRING version 10.5 [17] for generating protein-protein interactions and exploring the biological processes the genes are involved in. A threshold of minimum required interaction score was taken at 0.4 and

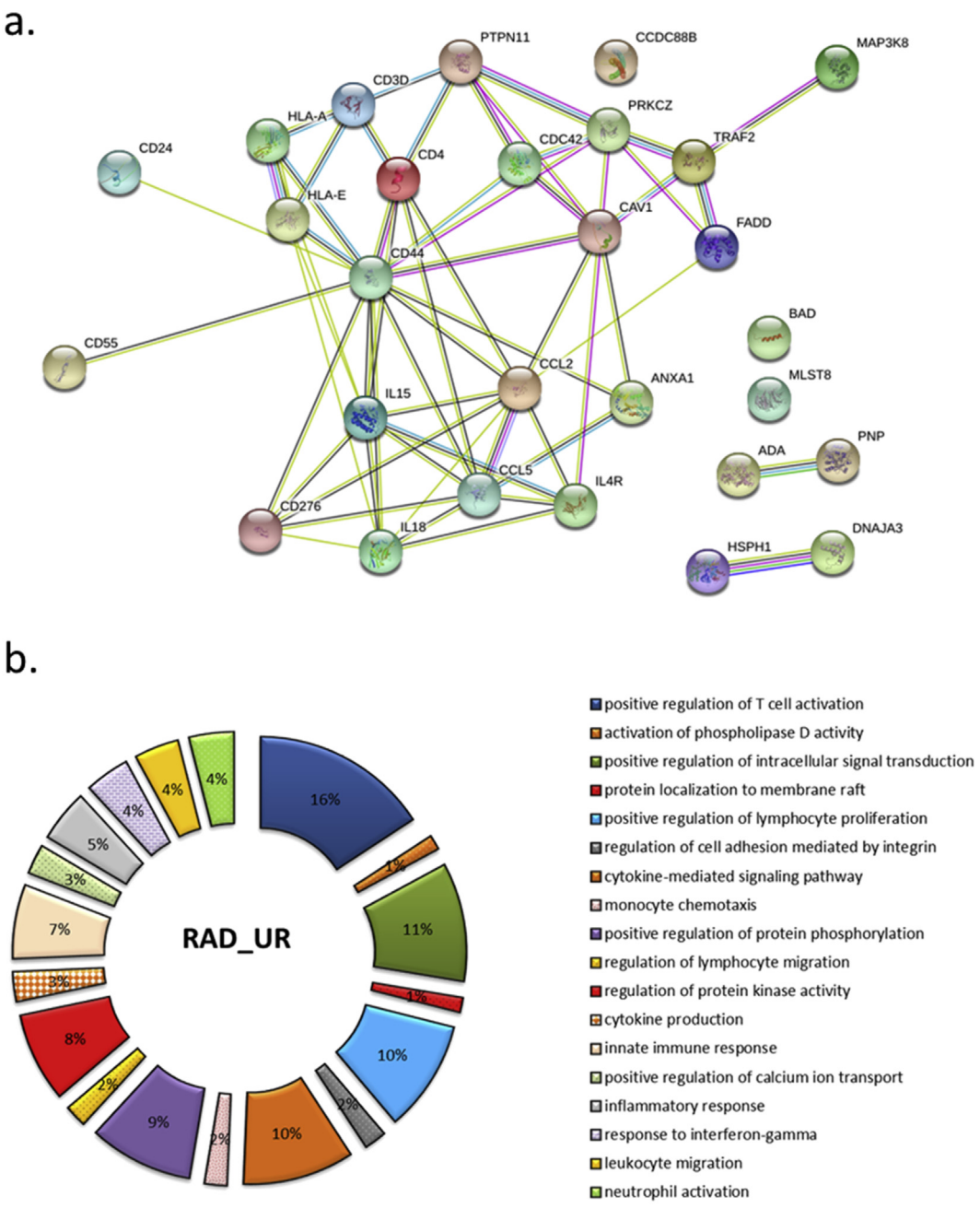

Fig. 2. List of genes/proteins determined in leukocyte-cell adhesion ontology for differentially upregulated genes/proteins in irradiated cells (RAD_UR) from DAVID were entered into STRING software and generated **(a)** Protein-protein interaction network with PPI enrichment p-value 8.88E-16 and average local clustering coefficient 0.566; **(b)** Biological processes having FDR ≤ 0.01 with quantity of genes/proteins involved in the process expressed as percentage over the number of genes/proteins involved in leukocyte-cell adhesion ontology for differentially upregulated proteins in irradiated cells (RAD_UR).

FDR (false discovery rate) values were taken at ≤ 0.01 for significant biological processes.

2.7. Identification of the genes responsible for the molecular switch and their network formation

Genes upregulated for leukocyte adhesion pathways from RAD samples were run against differentially upregulated genes from R + A samples and those upregulated for substrate adhesion pathways from R + A samples were run against differentially upregulated genes of RAD samples using jVenn [18]. Similarly, substrate adhesion pathways genes downregulated in RAD samples were run against differentially downregulated genes of R + A samples. Genes found through Venn diagram were analysed by Heatmapper [19] for expression based clustering using average linkage and Euclidean distance for measurement. The selected genes were then examined by GeneMANIA [20] for co-expression based networks. Extensive literature survey of the selected genes was carried out to sort out the genes essential for the changes in the pathways.

2.8. Statistical analysis

All experiments were conducted in triplicates with nonirradiated cells without treatment with amifostine taken as control for the study. Student's t-test p-value among replicates was calculated in comparison to control.

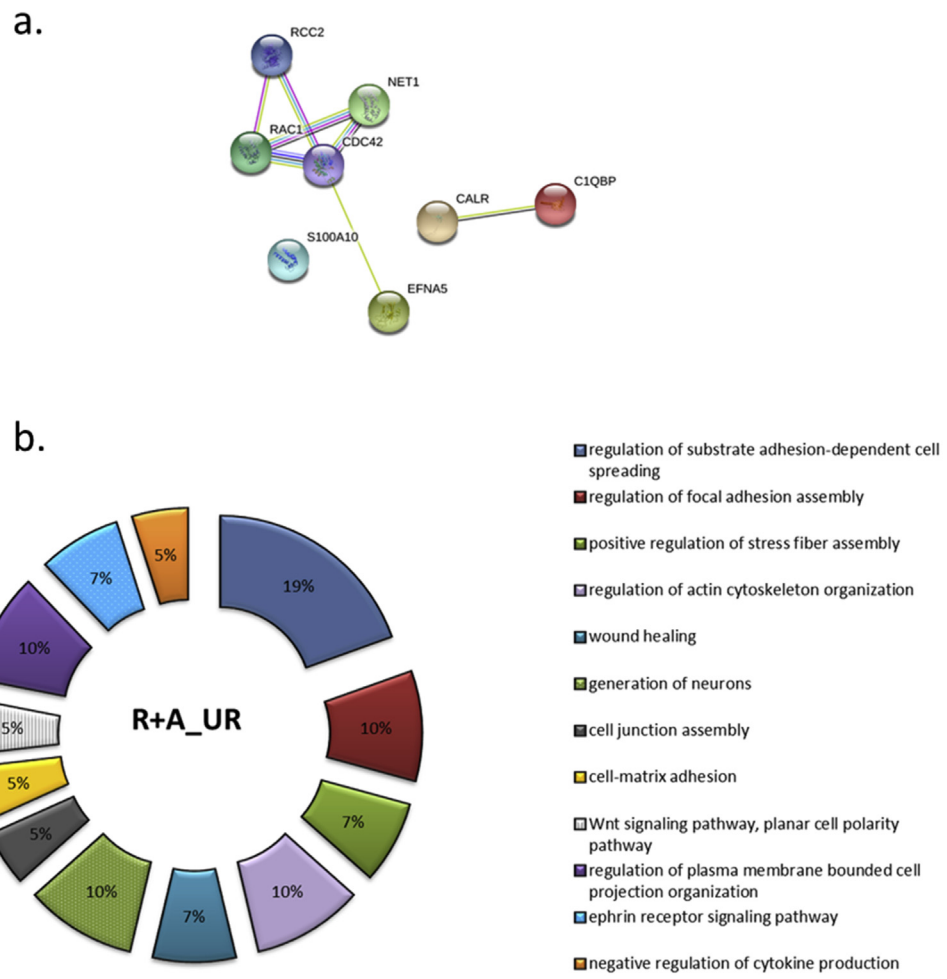

Fig. 3. List of genes/proteins determined in substrate-cell adhesion ontology for differentially upregulated genes/proteins in amifostine pretreated irradiated cells (R + A_UR) from DAVID were entered into STRING software and generated **(a)** Protein-protein interaction network with PPI enrichment p-value 3.05E-04 and average local clustering coefficient 0.75; **(b)** Biological processes having FDR ≤ 0.01 with quantity of genes/proteins involved in the process expressed as percentage over the number of genes/proteins involved in substrate-cell adhesion ontology for differentially upregulated proteins in amifostine pretreated irradiated cells (R + A_UR).

3. Results

3.1. Gene ontology and enrichment analysis

After applying the selection criteria of -2.0 ≥ FC ≥ 2.0 and p-value ≤ 0.05, AMF samples didn't show differentially expressed genes, whereas among 58203 probes analysed, 2216 and 1962 number of genes were differentially upregulated (UR) and 3182 and 3385 number of genes were differentially downregulated (DR) in RAD and R + A samples respectively. These genes were then uploaded into DAVID software and functional annotation clustering was performed.

Differentially upregulated genes in RAD samples (RAD_UR) as expected showed gene ontologies (GO) associated with negative regulation of telomere maintenance and chromosome organization; intrinsic apoptotic signalling pathways; collagen metabolism; fibril organization; T cell differentiation; T cell aggregation; leukocyte aggregation; response to hypoxia and peroxide, membrane budding; regulation of programmed cell death; metabolism of biomolecules like lipids, carbohydrates, proteins; myotube differentiation, etc. Differentially upregulated genes in R + A samples (R + A_UR), on the other hand, had ontologies like positive regulation of cell projection, NF-κB activity, neuron projection, nitric oxide biosynthesis; DNA biosynthesis, etc.

Differentially downregulated genes in RAD samples (RAD_DR) belonged to ontologies such as cell projection morphogenesis; cell migration; cell polarity; regulation of MAPK cascade; mitotic DNA damage checkpoint; regulation of intracellular signal transduction; positive regulation of nucleobase containing compound metabolism; endothelium development; exocytosis; synaptic vessel transport; fibroblast migration, etc. whereas genes downregulated in R + A samples (R + A_DR) belonged to ontologies like stress activated protein kinase signalling; apoptotic signalling; regulation of synaptic plasticity; regulation of myelination; Notch signalling; programmed cell death; actin cytoskeleton organization; positive regulation of cellular senescence; response to IL18, etc.

Among all the GO terms, those related with cell adhesion including both leukocyte-cell adhesion and substrate-cell adhesion, had 58 genes upregulated in differentially expressed genes in RAD samples (Fig. 1) whereas R + A samples showed only 8 upregulated genes affected for adhesion ontology. For differentially downregulated genes RAD samples showed 48 genes and R + A had no genes in any adhesion related ontology at all.

3.2. Protein Protein interaction (PPI)

On further dissection gene ontologies for cell adhesion exhibited leukocyte-cell adhesion in upregulated RAD samples and substrate-cell adhesion in upregulated R + A and in downregulated RAD samples. These ontologies were studied for protein interactions by using STRING. Average local clustering coefficient was 0.566 and 0.75 for upregulated genes in RAD (Fig. 2a) and R + A cells (Fig. 3a) with PPI enrichment p-value 8.88E-16 and 3.05E-04 respectively. Analysis of biological processes with FDR ≤ 0.01, showed that the radiation induced upregulated genes were primarily involved in regulation of immune processes (Fig. 2b and Supplementary Table 1) such as T cell activation; lymphocyte and leukocyte migration; cytokine mediated signalling; leukocyte activation and differentiation; innate immune response; adaptive immune response; regulation of protein phosphorylation; response to lipid; monocyte chemotaxis and inflammatory

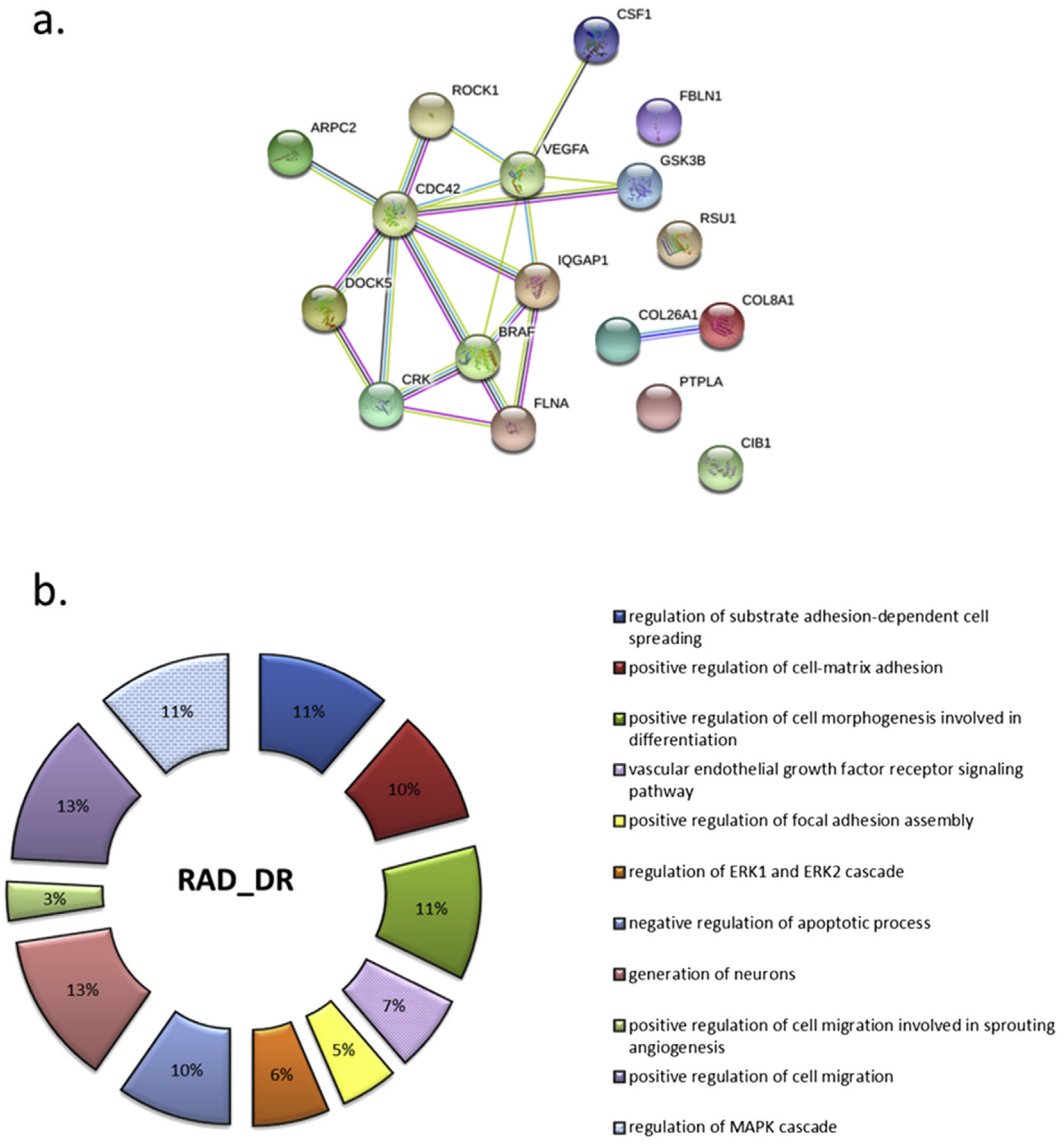

Fig. 4. List of genes/proteins determined in substrate-cell adhesion ontology for differentially downregulated genes/proteins in irradiated cells (RAD_DR) from DAVID were entered into STRING software and generated **(a)** Protein-protein interaction network with PPI enrichment p-value 2.78e-06 and average local clustering coefficient 0.548; **(b)** Biological processes having FDR ≤ 0.01 with quantity of genes/proteins involved in the process expressed as percentage over the number of genes/proteins involved in substrate-cell adhesion ontology for differentially downregulated proteins in irradiated cells (RAD_DR).

response; leukocyte cell-cell adhesion; cholesterol homeostasis; calcium ion transport; integrin mediated cell adhesion; macromolecule metabolism, etc.

Differentially upregulated genes in R + A cells were instead focussed (Fig. 3b and Supplementary Table 2) on cell-matrix adhesion, substrate adhesion dependent cell spreading; focal adhesion complex; cell migration; Wnt signalling for cell polarity; VEGFR signalling pathway; cell junction assembly; wound healing; plasma membrane bounded cell projection organization and nervous system development.

With average local clustering coefficient of 0.548 and PPI enrichment p-value of 2.78e-06 downregulated genes in RAD cells (Fig. 4a) exhibited biological processes present in upregulated genes of R + A cells like (Fig. 4b and Supplementary Table 3) cell-matrix; substrate adhesion dependent cell spreading; focal adhesion complex; regulation of angiogenesis; VEGFR signalling pathway; cell morphogenesis, etc.

3.3. Identification of the genes responsible for the molecular switch

To determine the genes responsible for the transformation in the adhesion processes, genes upregulated for leukocyte-cell adhesion were cross-referenced for their presence in differentially upregulated genes in R + A samples and genes upregulated for cell-substrate adhesion were cross-referenced for their presence in differentially upregulated genes in RAD samples. Similarly genes downregulated for cell-substrate adhesion were cross-referenced for their presence in differentially downregulated genes in R + A samples. It was seen that 7 genes- BAD, CCL2, CD3D, DNAJA3, IL4R, MAP3K8 and PRKCZ were present in upregulated genes of leukocyte-cell adhesion pathway in RAD samples but were absent in R + A treatment whereas RAC1 and EFNA5 were present in cell-substrate adhesion pathway in R + A treatment but absent in RAD treatment (Fig. 5a). Similarly 4 genes- BRAF, ROCK1, FLNA and FBLN1 were found in downregulated genes in RAD group for cell-substrate adhesion pathway but absent in downregulated genes of R + A group (Fig. 5b). Expression based heatmap (Fig. 6) showed the difference in expression levels of the selected genes between irradiated (RAD) and amifostine pretreated irradiated cells (R + A). Functional significance of the genes (Table 1) was studied in literature and described in relevance to endothelial cells.

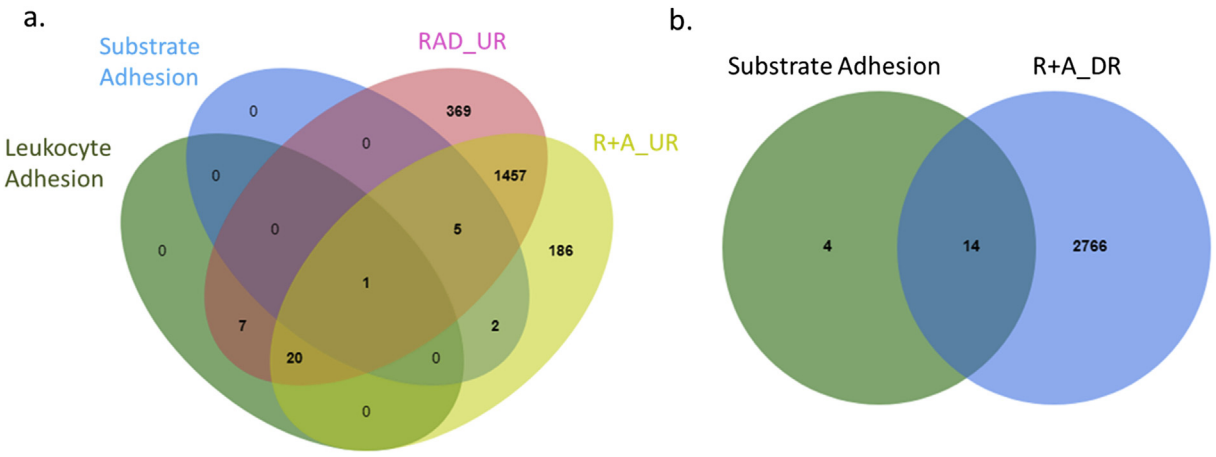

Fig. 5. Venn diagram obtained from jVenn for **(a)** differentially upregulated genes in leukocyte cell adhesion ontology from irradiated samples (RAD) and substrate cell adhesion ontology from amifostine pretreated irradiated samples (R + A) compared with differentially upregulated genes in R + A_UR and RAD_UR samples respectively; **(b)** differentially downregulated genes for substrate cell adhesion ontology from irradiated samples (RAD) samples compared with differentially downregulated genes in R + A_DR samples.

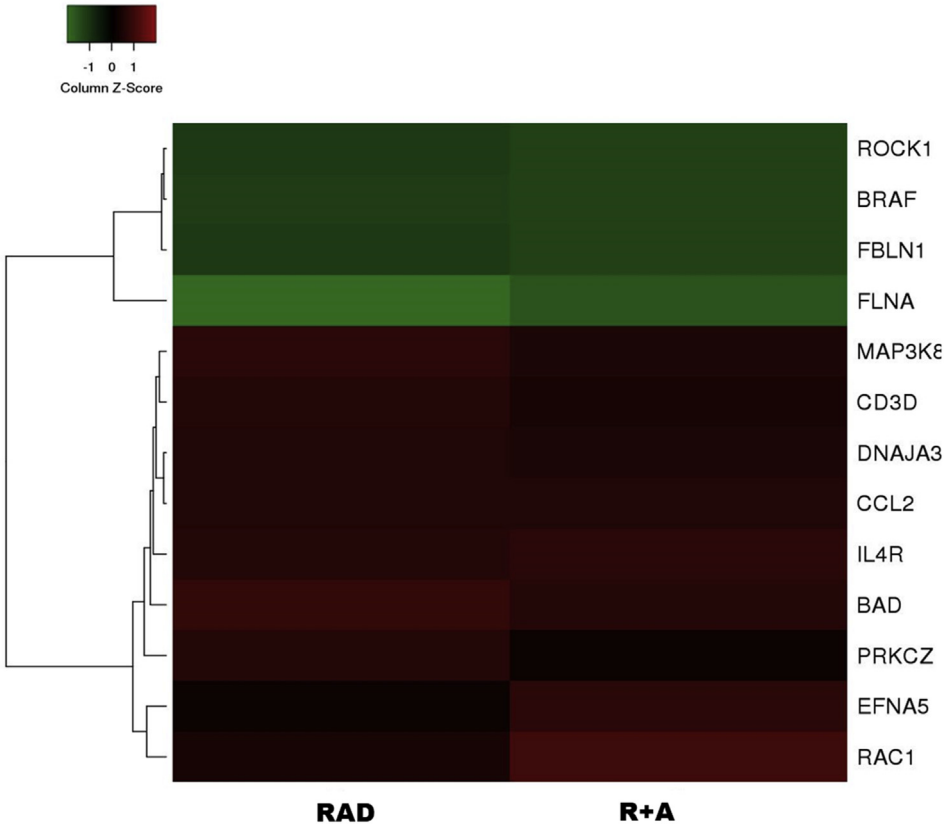

Fig. 6. Expression based heatmap prepared using Heatmapper between irradiated (RAD) and amifostine pretreated irradiated cells (R + A) using average linkage and Euclidean distance for measurement for the genes selected through venn diagram analysis.

3.4. Network for the selected genes

Investigation of the co-expression based network of the selected genes formed by GeneMANIA (Fig. 7) revealed that IL4 through its interaction with IL4R upregulates VCAM1 and CCL2 as well as activates LIM kinase and cofilin through Wnt5 signalling to increase vessel permeability and induces adhesion of lymphocytes to endothelial cells [44]. MAP3K8 deficient microglia has been seen to express low levels of CCL2 and thereby decreased leukocyte migration [45] thus affirming that activation of CCL2 involves MAP3K8 as a mediator molecule. CD3D interacts with IL4R to regulate hematopoietic lineage formation [46] and with PRKCZ to regulate hematopoiesis and calcium induced T-lymphocyte apoptosis [47] but its role in adhesion and migration pathways is not yet clear. Similarly BAD is known for its proapoptotic activity but no evidence of direct involvement of BAD in endothelial cell specific responses has been found although it has been seen that ERK5, crucial for endothelial cell differentiation in response to VEGF, regulates VEGF-mediated phosphorylation of BAD [48]. Known for its role in adhesion, PRKCZ when downregulated, decreased BAD phosphorylation [49]. PRKCZ on the other hand regulates cell polarity during endothelial cell migration and sprouting by polarized integrin dependent Rac and Cdc42 activity. DNAJA3 is also known to play a role

Table 1
Functional role of the genes selected through venn diagram analysis.

S·NO.	GENES	FUNCTIONAL ROLE
1.	BAD (Bcl-2 Agonist of Cell Death)	BAD is known for its proapoptotic activity. In its dephosphorylated state it forms a complex with antiapoptotic Bcl-2 thus inactivating it whereas phosphorylation by Akt/protein kinase B, refrains its interaction with Bcl-2 resulting in Bcl-2 mediated suppression of Bax induced apoptosis.
2.	CCL2 (C-C motif chemokine ligand 2)	CCL2 stimulated ECs showed increased MLC phosphorylation leading to cell retraction and vascular leakiness [21]. CCL2 have been reported to activate PKCα resulting in redistribution of tight junction protein [22,23].
3.	IL4R	IL4R when binds to its ligand IL4, activates LIM kinase causing cytoskeleton rearrangement and reversible increase in endothelial layer permeability [24,25].
4.	MAP3K8 (Mitogen activated protein kinase kinase kinase 8)	MAP3K8 depletion causes decrease in monolayer permeability and VEGF induced tubulogenesis and endothelial-leukocyte interaction [26]. It causes proliferation, migration and capillary tube formation in ECs [27].
5.	PRKCZ (Protein kinase C zeta)	Ang-1 induces colocalization of PRKCZ and β-catenin at the leading edge of migrating ECs for vessel sprouting [28]. Loss of PRKCZ affects apical polarization and lumenogenesis in vessels [29].
6.	CD3D	CD3D encodes delta subunit of CD3 involved in T-cell receptor/CD3 complex. It contains immunoreceptor tyrosine base activation motif which becomes phosphorylated on TCR-CD3 interaction leading to T cell activation.
7.	DNAJA3 (DNAJ heat shock protein family member 3)	DNAJA3 plays crucial role in protein folding and degradation. It stimulates ATPase activity of Hsp70 chaperone in mitochondria and is thus important for cell growth, proliferation and apoptosis. It has been known to affect cytochrome c and caspase 3 required in apoptosis.
8.	Rac1(Rac family small GTPase 1)	Rac1 belongs to Ras family of small GTP binding proteins and induces lamellopodia and filopodia required for cell spreading. Integrin mediated cell-ECM interaction activates Rac1 which mediates cytokine stimulated redox dependent NFκB activation leading to ICAM1 upregulation [30]. It has been seen to localize with E cadherin at the site of cell-cell contact and it also regulates protrusion of membrane ruffles for cell-substrate adhesion [31].
9.	EFNA5 (Ephrin A5)	EFNA5 is GPI linked protein and interacts with EphB2 receptor tyrosine kinase which is known to increase cell motility as well as cell contraction and respreading [32,33].
10.	FBLN1 (Fibulin-1)	FBLN1 binds to fibronectin, laminin, collagen and fibrinogen thus contributing to ECM architecture by being a component of basement membrane [34]. Since it can bind to fibrinogen it is also seen incorporated in clots. Fibulin-1 deficient mice with abnormal capillary development and blood leakage causing massive haemorrhages have been reported [35].
11.	ROCK1 (Rho associated coiled coil containing protein kinase 1)	ROCK1 is a serine/threonine kinase activated by Rho by phosphorylation and in turn phosphorylates and activates MLC resulting in increased cell motility [36]. ROCK1 knockdown decreases adhesion to fibronectin, wound closure and EC migration [37]. ROCK1 activation of FAK is required for focal adhesion in EC following VEGF treatment.
12.	BRAF (v-Raf murine sarcoma viral oncogene homolog B)	Loss of BRAF causes relocalization of VE-cadherin and decreased vessel permeability [38]. BRAF deficient embryos show increased endothelial precursors, enlarged vessels and apoptosis of differentiated ECs [39].
13.	FLNA (Filamin A)	FLNA promotes orthogonal branching of actin filaments and links them to membrane glycoprotein [40]. It anchors cells to ECM, regulates focal adhesion (FA) turnover, maintains cytoskeletal integrity and controls cell spreading [41,42]. FLNA null vascular ECs display defects in cell-cell contact and adherens junction leading to disorganized vasculature, misshapen ECs and defective vessels [43]. Its deficiency alters VE-cadherin associated with adherens junction.

in MUSK signalling at neuromuscular junctions [50,51] therefore up-regulation of DNAJA3 in leukocyte adhesion pathways seems intriguing and need to be further explored. EFNA5 interacts with its receptor and induces membrane ruffling, endocytosis and cell repulsion mediated by Rac1 [52]. FLNA regulates cytoskeleton by R-Ras activation [53] which is known to signal though BRAF-MEK-ERK pathway and also been observed to mediate integrin activation by GTPase Rap1 [54]. FLNA also regulates Rac1 activity and interacts with ROCK1 to regulate cytoskeletal organization [55]. Since ROCK1 silencing leads to higher elimination of melanocytes when combined with BRAF inhibition [56], involvement of BRAF in angiogenesis [57] has been suggested. Despite being a part of ECM architecture, FBLN1 has not been seen to interact with any molecules in the network.

In summation, it was seen that BAD and BRAF interact with other molecules but only as a means to regulate cell survival and proliferation. Not much information on CD3D and DNAJA3 was explored in terms of adhesion and migration. Thus BAD, BRAF, CD3D and DNAJA3 can be ruled out as significant for adhesion. Lack of interaction of FBLN1 to any other molecules resulted in no associated networks. IL4R, EFNA5 and CCL2 networks placed them far upstream on signalling pathways operating via numerous effectors whereas FLNA and PRKCZ seemed to be terminal molecules regulating cell-cell contact and cell polarity respectively. MAP3K8 on the other hand was positioned at the centre by being a mediator of upstream inflammatory molecules like CCR2, IL1R, etc. and activates all MAPK signalling pathways downstream like ERK, ERK5, p38 and JNK thus regulating actin cytoskeleton [58]. It is well known that ROCK1 regulates actin filament by activating MLCK in response to cell adhesion molecules thus affecting cell-cell contact. Rac1 has been one of the key molecules involved in all adhesion pathways such as focal adhesion, actin cytoskeleton, cell polarity etc. Therefore it can be deduced that FLNA, PRKCZ, MAP3K8, ROCK1 and RAC1 are the prime manipulators of adhesion pathways. Since RAC1 colocalizes with E-cadherin and functions downstream of integrin signalling, it affects both cellular junctions and cell adhesion. Rho GTPases through its downstream effector ROCK1 have been implicated in regulation of E-selectin and modulation of cell-cell junction via actin cytoskeleton in response to VCAM1, ICAM1 signalling and therefore is involved in rolling, adhesion as well as junctional processes comprising extravasation. FLNA has been seen to alter adherens junction whereas MAP3K8 and PRKCZ has been more inclined towards modulating cell-cell adhesion. It can thus be said that MAP3K8 and PRKCZ increase leukocyte activation and migration whereas RAC1, ROCK1 and FLNA twitch the pathways towards cell-ECM interaction and cell-cell contact.

4. Discussion

Irradiation severely compromises the functional integrity of endothelial vessels required to maintain homeostasis between blood and the underlying tissue. This not only includes DNA damage, cellular apoptosis and loss of endothelial cells thus increasing the vessel permeability, but also involves changes in molecular expression that progressively leads to tissue damage. Although gene profiling studies have been performed on endothelial cells with emphasis on cell cycle, apoptosis, DNA damage and repair, angiogenesis and cell proliferation in response to radiation [59–61], there has been no gene expression study conducted to assess the radiation induced changes in adhesion pathways that are primary reason behind tissue damage. In addition, all the gene expression studies were mainly conducted with human umbilical vein endothelial cells (HUVECs) as *in vitro* model. But it has been long established that endothelial cell phenotypes vary between different organs in structure and function [62], which might lead to different response by different tissues to the same stimuli. Thus it is

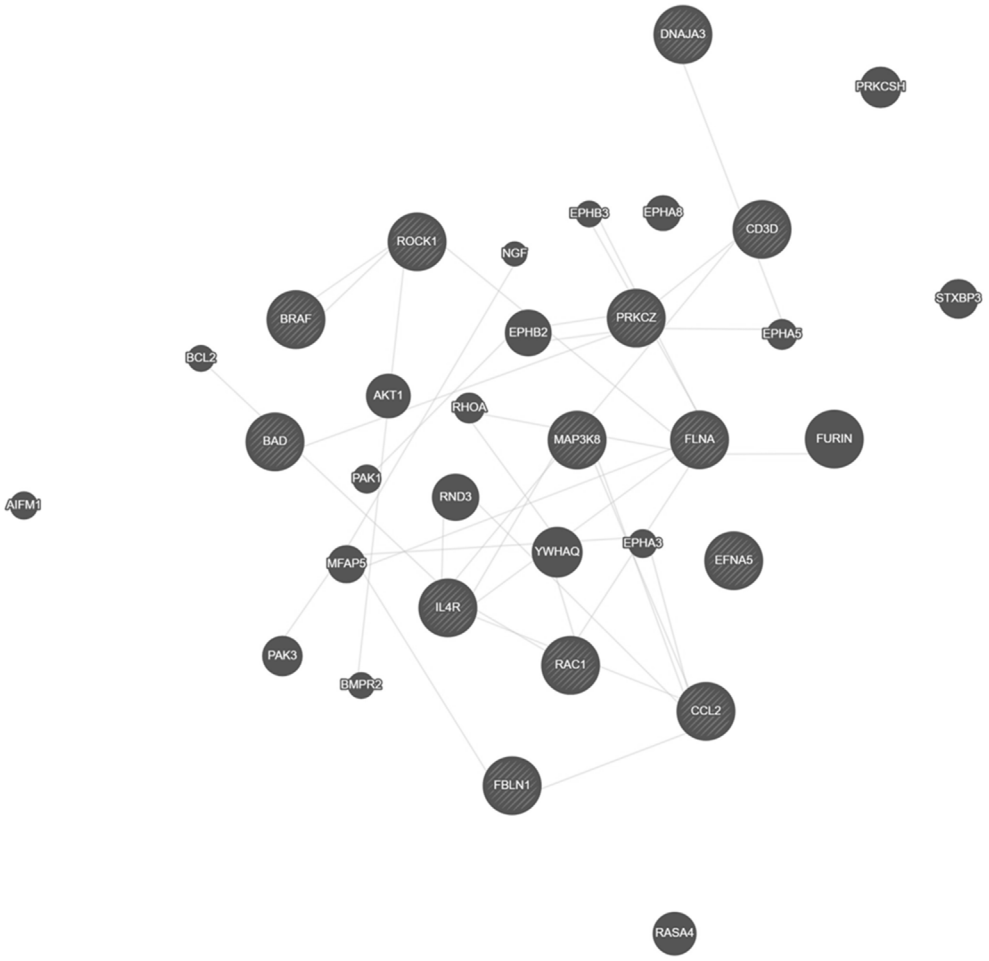

Fig. 7. Co-expression based interaction network of selected genes obtained from GeneMANIA for the genes selected through venn diagram analysis. Selected genes are indicated with stripes.

important to study endothelial cell associated with each organ separately especially bone marrow endothelial cells since they are the major part of stem cell niche. Therefore we have studied the changes in signalling pathways of irradiated cells and shift in these pathways when administered with amifostine preceding radiation exposure.

Our study showed that adhesion related ontologies had maximum gene count in differentially upregulated genes of RAD samples and none were found in downregulated genes in R + A samples. Gene ontologies activated in irradiated cells included leukocyte adhesion whereas radioprotected cells showed the ontology of substrate dependent cell spreading similar to that of downregulated genes in RAD samples. Several biological processes were found induced among respective adhesion ontology genes with STRING. For example, interaction of leukocyte to endothelial cells and their subsequent integrin mediated adhesion followed by protein phosphorylation, MAPK cascade, calcium ion transport and cholesterol homeostasis leading to migration of leukocyte into vascular bed as well as secretion of cytokines to activate leukocytes and lymphocytes were all upregulated in irradiated endothelium. Nitric oxide (NO) biosynthesis has been upregulated in R + A treated endothelial cells since NO has been known to inhibit platelet aggregation and regulate leukocyte adhesion as well as inhibit transcription of genes implicated in atherosclerosis [63–67] thus rendering the endothelium anti-adhesive and anti-coagulative. Processes like regulation of cell polarity, formation of cell protrusions, appearance of focal adhesion complex and migration of endothelial cells are all characteristics of angiogenesis. This kind of cell motility is the result of interaction with the substrate or ECM, chemotactic signalling by growth factors like VEGF and cell-cell contact [68] which were found in upregulated genes in R + A and downregulated genes in RAD samples.

Genes for adhesion ontology found in one treatment were cross-referenced with the DEGs in opposite treatment and 13 genes- BAD, CCL2, DNAJA3, CD3D, IL4R, MAP3K8, PRKCZ, RAC1, EFNA5, BRAF, FLNA, FBLN1 and ROCK1 were identified to be responsible for the switch and further studied. It was observed that BAD, BRAF, CD3D, DNAJA3, FBLN1, EFNA5, IL4R are not crucial whereas FLNA, PRKCZ, MAP3K8, ROCK1 and RAC1 are found in various adhesion pathways including cell polarity, actin cytoskeleton, membrane ruffles, cell-matrix adhesion, leukocyte activation, leukocyte-cell adhesion etc. and therefore can be regarded as a molecular switch that control transformation of cell-substrate adhesion to cell-leukocyte adhesion. MAP3K8 and PRKCZ were seen involved in cell adhesion, FLNA in cell junction, RAC1 in cell junction and adhesion whereas ROCK1 is a part of rolling, adhesion and cell junction.

These results alter the initial hypothesis that adhesion pathways may get inactivated when using a radioprotectant before radiation exposure and endothelial cells might return to their ground state level. Instead the study shows that adhesion pathways remain activated in both irradiated and radioprotected conditions, although their nature remains different. Radiation exposure elicits adhesion pathways that lead to leukocyte adhesion and migration, activation of immune cells, response to oxidative stress and various cytokines. These pathways seem to shift in their nature when radioprotected conditions are taken into account and concentrate towards DNA damage and repair, adhesion to substrate and cell spreading, formation of focal adhesion

complexes that link the cells with extracellular matrix. So it can be concluded that radiation activated adhesion pathways lead to immune cell activation and their migration to underlying tissue causing tissue damage. On the contrary, these adhesion pathways shifts towards DNA repair, cell-ECM contact, cell-substrate adhesion, cell spreading that leads to repair of endothelial monolayer and reconstitutes the vessel wall integrity.

5. Conclusion

Here we report the activated adhesion pathways after radiation exposure on BMECs and altered adhesion pathways on treatment with the radioprotectant. Our study substantiates that leukocyte adhesion pathways mainly get induced after irradiation and these adhesion pathways shifts from leukocyte-cell interactions to substrate cell interaction probably leading to endothelial cell layer repair. If the genes regulating these adhesion pathways can be found, their expression can be manipulated to control the intensity of adhesiveness of endothelial cell surface and thus can be used to reduce several diseases induced by radiation or inflammation like rheumatoid arthritis, asthma, autoimmune diseases, etc. We have identified a set of genes that can modulate these pathways but an additional experimental validation is required.

CRediT authorship contribution statement

Swati Gupta: Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Software, Validation, Visualization, Writing - original draft, Writing - review & editing. **Gurudutta Gangenahalli:** Conceptualization, Writing - original draft, Funding acquisition, Investigation.

Declaration of competing interest

The authors declare no competing financial interests.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.lfs.2019.116981.

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