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TOPIC HIGHLIGHT

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Significance of endothelial dysfunction in the pathogenesis of early and delayed radiation enteropathy

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Abstract

This review summarizes the current state of knowledge regarding the role of endothelial dysfunction in the pathogenesis of early and delayed intestinal radiation toxicity and discusses various endothelial-oriented interventions aimed at reducing the risk of radiation enteropathy. Studies published in the biomedical literature during the past four decades and cited in PubMed, as well as clinical and laboratory data from our own research program are reviewed. The risk of injury to normal tissues limits the cancer cure rates that can be achieved with radiation therapy. During treatment of abdominal and pelvic tumors, the intestine is frequently a major dose-limiting factor. Microvascular injury is a prominent feature of both early (inflammatory), as well as delayed (fibroproliferative) radiation injuries in the intestine and in many other normal tissues. Evidence from our and other laboratories suggests that endothelial dysfunction, notably a deficiency of endothelial thrombomodulin, plays a key role in the pathogenesis of these radiation responses. Deficient levels of thrombomodulin cause loss of vascular thromboresistance, excessive activation of cellular thrombin receptors by thrombin, and insufficient activation of protein C, a plasma protein with anticoagulant, anti-inflammatory, and cytoprotective properties. These changes are presumed to be critically involved in many aspects of early intestinal radiation toxicity and may sustain the fibroproliferative processes that lead to delayed intestinal dysfunction, fibrosis, and clinical complications. In conclusion, injury of vascular endothelium is important in the pathogenesis of the intestinal radiation response. Endothelial-oriented interventions are appealing strategies to prevent or treat normal tissue toxicity associated with radiation treatment of cancer.

Key words: Endothelial cells; Thrombomodulin; Proteinase-activated receptors; Radiation injuries; Radiation enteropathy

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INTRODUCTION

There are currently more than 10 million cancer survivors in the United States[1]. The exponential increase in the cancer survivor population has led to a stronger focus on reducing treatment-related side effects, thus prompting a more proactive approach aimed at acquiring a better understanding of the molecular and cellular basis of treatment-related side effects, and at developing interventions to ameliorate or prevent long term toxicities of cancer therapy.

Approximately 70% of all cancer patients receive radiation therapy at some point during the course of their disease and radiation therapy plays a critical role in 25% of all cancer cures[2]. Recent advances in treatment delivery, such as the development of dose-sculpting techniques, have led to an overall reduction in normal tissue exposure during radiation therapy. Nevertheless, normal tissue radiation toxicity remains the single-most important dose-limiting factor in radiation therapy and a major obstacle to uncomplicated cancer cures.

More than 200 000 patients in the United States undergo localized radiation therapy for abdominal, pelvic, and retroperitoneal malignancies each year. During

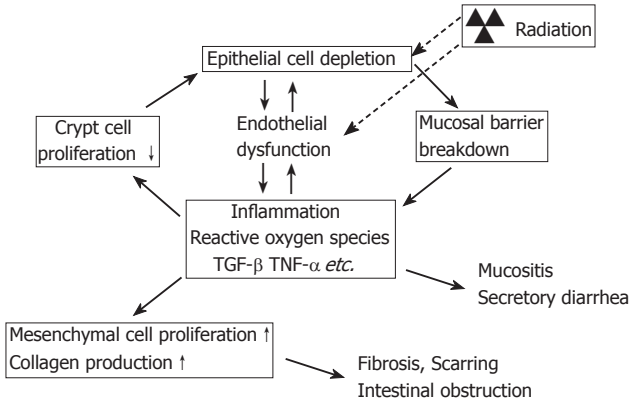


Figure 1 Model of interaction between epithelial and endothelial radiation injury in the intestine demonstrating how endothelial dysfunction may exacerbate the early intestinal radiation response and "drive" the cycle of chronicity of intestinal radiation fibrosis. Radiation causes epithelial crypt cell death, leading to insufficient replacement of the villus epithelium, and breakdown of the epithelial barrier that normally separates intestinal tissue from the intraluminal contents of the intestine. Simultaneously, radiation causes endothelial dysfunction, notably loss of thromboresistance and increased expression of chemokines and adhesion molecules. The combination of loss of epithelial barrier function and endothelial dysfunction enhances the post-radiation inflammatory response, inhibits restitution of the epithelium, and promotes extracellular matrix deposition.

treatment of such tumors, the bowel is almost invariably exposed and the risk of intestinal radiation injury (radiation enteropathy) is often the most important dose-limiting factor.

Radiation enteropathy is classified as early (acute) or delayed (chronic). Early radiation enteropathy occurs during or shortly after radiation therapy. It is a consequence of death of rapidly proliferating crypt cells, resulting in epithelial barrier breakdown and mucosal inflammation (radiation mucositis). Delayed radiation enteropathy, by convention, occurs three months or later after radiation therapy. Chronic radiation enteropathy is characterized by vascular sclerosis and progressive intestinal wall fibrosis, leading to intestinal dysfunction (e.g., dysmotility or malabsorption) and structural injury (e.g., stricture formation, fistulas, or perforation). In addition to radiation-induced cell death, radiation enteropathy is the result of a complex interplay among a plethora of pathophysiological processes, including activation of the coagulation system, inflammation, epithelial regeneration, tissue remodeling and collagen deposition. These processes are orchestrated by a large number of cell types and interacting molecular signals, including cytokines and growth factors, as well as various molecules on the endothelial cell surface[3]. Functional perturbation of these endothelial cell molecules is collectively referred to as endothelial dysfunction.

ENDOTHELIAL DYSFUNCTION IN EARLY AND DELAYED RADIATION ENTEROPATHY

Effects of ionizing radiation on the vascular endothelium

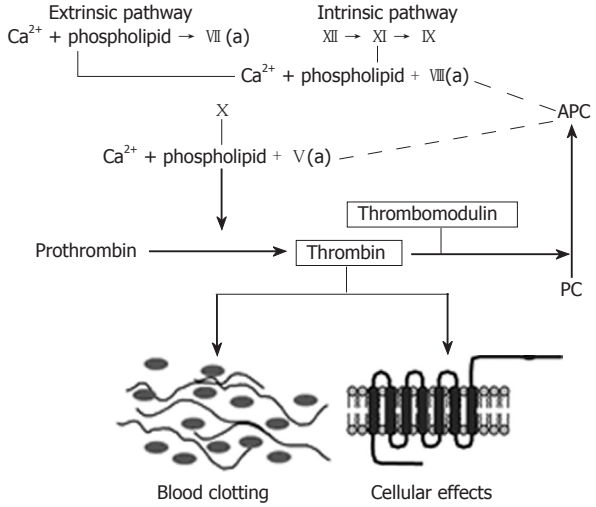
Endothelial cells form the inner lining of blood vessels and cover a total surface area of 4000-7000 m^2[4]. Endothelial cells are highly dynamic and participate in a multitude of physiological functions, including maintenance of blood fluidity, control of vasomotor tone, trafficking of cells and nutrients, and growth of new blood vessels[5]. Under normal conditions, endothelial cells maintain an antithrombotic and anticoagulant balance by exerting molecular control of platelet aggregation, coagulation and fibrinolysis[6].

An increasing body of evidence shows that injury of the microvasculature plays a central role in early and delayed radiation responses in many normal tissues, including the intestine. Notably, microvascular injury may be responsible for the unique self-perpetuating nature of chronic radiation fibrosis[7-13]. A model depicting how endothelial cell dysfunction may contribute to and sustain post-radiation inflammatory and fibroproliferative responses in the intestine is shown in Figure 1.

The high radiation sensitivity of the microvasculature is to a large extent attributable to the endothelial cells[14]. Radiation induces a plethora of morphological and functional alterations in endothelial cells, including apoptosis, detachment from the basement membrane, and increased endothelial permeability, resulting in fibrin deposition in the interstitial space[15,16].

The role of endothelial apoptosis in early intestinal radiation toxicity, particularly in the so-called acute gastrointestinal radiation syndrome, has been a much debated issue for a number of years. The debate originated from reports that mice deficient in the enzyme acid sphingomyelinase are protected from radiation-induced endothelial cell apoptosis, and that these mice also exhibit decreased levels of crypt cell apoptosis and decreased lethality after total body irradiation[17]. Because endothelial cell apoptosis, but not apoptosis of the crypt epithelium, is sphingomyelin-dependent, the interpretation of this finding, together with a substantial body of additional supportive evidence, was that endothelial cell apoptosis appears to be a major contributor to early intestinal radiation toxicity and that there may be a causal relationship between endothelial cell apoptosis and crypt cell apoptosis. There has, however, been considerable controversy related to the extent and significance of endothelial apoptosis in the intestinal microvasculature after radiation exposure, and to whether or not there is a direct relationship between endothelial apoptosis and apoptosis in the crypt epithelium[18]. Despite this controversy, it may be possible to reconcile these seemingly contradictory findings. It is well known from other areas of gastrointestinal pathophysiology that genetic manipulations or pharmacologic interventions that preserve the intestinal microcirculation after an insult have a protective effect on the gut epithelium and the intestinal mucosa. Therefore, it is conceivable that radiation-induced endothelial cell apoptosis may be the bellwether, or "tip of the iceberg" that indicates a state of dysfunction of the intestinal microvasculature, and that it is the state of endothelial dysfunction that adversely affects the radiation tolerance and/or repair capacity of the crypt epithelium.

Loss of thromboresistance is a major feature of endothelial dysfunction after exposure to ionizing radiation. Radiation induces adhesion and aggregation of platelets and development of platelet-fibrin thrombi[19-22],



Figuge 2 The coagulation cascade. Simplified diagram of the coagulation "cascade" with the intrinsic, extrinsic, and common pathways. Note how thrombomodulin, located on the luminal surface of endothelial cells, forms a complex with thrombin, which is converted from a pro-coagulant to an anticoagulant and how activated protein C (APC) limits thrombin generation by feed-back into the intrinsic and common coagulation pathways. See text for further details.

as well as adhesion of inflammatory cells to the endothelium[23-25] with subsequent perivascular leukocyte infiltration. The molecular basis underlying the loss of endothelial thromboresistance is complex and includes increased expression of tissue factor[26,27], von Willebrand factor (vWF)[28-30], and platelet activating factor (PAF)[31]; reduction in fibrinolytic activity[32-34]; and radiation-induced reduction in the expression of prostacyclin (PGI2), the PGI2 receptor[35-37], and thrombomodulin (TM)[12,38]. Studies performed in our laboratory suggest that radiation-induced loss of TM may play a particularly important role in the pathogenesis of radiation enteropathy.

The thrombomodulin-protein C system

Endothelial TM is a transmembrane glycoprotein located on the luminal surface of endothelial cells in most normal blood vessels. TM forms a complex with thrombin, and essentially converts thrombin from a pro-coagulant to an anticoagulant by changing its substrate specificity. Thrombin, when in complex with TM, no longer cleaves fibrinogen to form fibrin and no longer activates cellular thrombin receptors, but instead activates protein C, thereby limiting further thrombin generation and counteracting thrombin's many coagulant, inflammatory, and fibroproliferative effects (Figure 2). In addition, both TM and activated protein C (APC) have important intrinsic anti-inflammatory properties.

Recent studies have demonstrated the importance of TM in attenuation of inflammatory responses in a variety of settings, such as, endotoxin-induced tissue damage, glomerulonephritis, and atherosclerosis[39-42]. One mechanism by which TM exerts its anti-inflammatory properties involves APC. APC inhibits leukocyte chemotaxis and leukocyte adhesion, suppresses inflammatory cytokine production, reduces endothelial cell apoptosis, and maintains endothelial cell barrier function[43-48]. In addition, recent studies have shown that TM has potent intrinsic anti-inflammatory properties by virtue of its N-terminal domain binding and inhibiting high mobility group box 1 protein (HMGB1)[49].

Clinical and preclinical studies performed in our laboratory have shown that radiation causes a striking (80%-90%) and sustained reduction in endothelial TM expression in intestinal microvasculature[12,50,51]. The reduction in TM appears to be due to a combination of direct oxidative damage [52,53], and down regulation of TM at the gene expression level by radiation-induced inflammatory cytokines such as interleukin 1 (IL1), tumor necrosis factor α (TNFα) and transforming growth factor β (TGFβ)[54-57], and increased release of TM from the endothelial cell membrane into the circulation (ectodomain shedding) by granulocyte proteinases and other inflammatory mediators[58].

Thrombin and cellular thrombin receptors

In the normal situation, thrombin is rapidly removed from the microcirculation by complex formation with TM. Local deficiency of TM, such as occurs after irradiation, leads to decreased thrombin clearance and insufficient protein C activation, resulting in accumulation of thrombin. Moreover, the expression of tissue factor, a critical initiator of thrombin generation, can also be triggered by radiation, both *in vitro* and *in vivo*[26,27]. Hence, radiation enhances thrombin generation both through the intrinsic and the extrinsic pathway.

Thrombin induces gap formation between endothelial cells, resulting in increased vascular permeability[59-62]. Consequently, thrombin may pass through the endothelial cell layer into the vessel wall and extravascular tissues. Studies performed in our laboratory show that radiation causes deposition of enzymatically active thrombin on the vascular endothelium, in the vascular wall of small arteries, as well as in the extravascular connective tissue[27]. Thrombin bound to extracellular matrix remains functionally active and able to generate fibrin and interact with surrounding cells[63,64]. We have demonstrated increased deposition of fibrin in irradiated intestine that co-localizes with enzymatically active thrombin[27].

Thrombin, in addition to its central role in coagulation, activates a variety of cell types including endothelial cells, smooth muscle cells, leukocytes, and platelets, thereby enhancing many inflammatory and fibroproliferative processes. For example, thrombin has chemotactic activity for monocytes and leukocytes and stimulates the migration of these cells to sites of injury[65]. Thrombin stimulates fibroblast chemotaxis[66], fibroblast proliferation[67,68], and fibroblast procollagen production[69]. Thrombin also enhances proliferation and migration of smooth muscle cells (SMC) and promotes SMC procollagen synthesis[70-72].

The cellular effects of thrombin are mediated by activation of cell surface thrombin receptors, proteinase activated receptors (PARs), a 4-member G-protein coupled receptor subfamily. Proteinase activated receptor 1 (PAR_1) is the biologically most relevant among the PARs[71-75]. Studies performed in our laboratory show that radiation upregulates PAR_1 expression in endothelium, SMC,

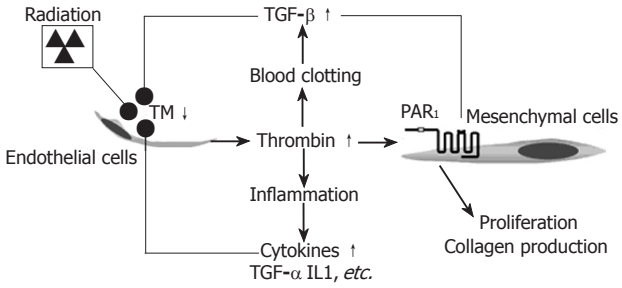


Figure 3 Proposed model linking radiation-induced endothelial dysfunction to chronic inflammation and progressive intestinal fibrosis via chronic PAR_1 activation. Radiation causes TM deficiency in endothelial cells, leading to insufficient "scavenging" of locally formed thrombin. Thrombin exerts pro-coagulant, pro-inflammatory, mitogenic, and pro-fibrogenic effects on mesenchymal cells (smooth muscle cells, fibroblasts, and myofibroblasts), as well as other cell types in the irradiated tissue. Feed-back by cytokines and other inflammatory mediators sustains the endothelial TM deficiency and thus contributes to the chronicity of radiation injury.

and myofibroblasts, particularly in areas of fibrosis[27]. Increased expression of PAR_1 in SMC may be particularly important in the context of intestinal wall fibrosis. This is because, in the intestine, SMC rather than fibroblasts are the predominant producers of collagen. Analogous to our observations in radiation enteropathy, upregulation of PAR_1 occurs in a number of other vascular disorders, including neointima formation after mechanical injury[73], as well as in response to injury-related cytokines and growth factors. Figure 3 depicts a model for how deficient levels of TM after radiation exposure, with subsequent increased thrombin formation and upregulation of PAR_1, may contribute to and sustain inflammatory and fibroproliferative responses in irradiated tissues. Consistent with this model, *in vivo* studies performed in our laboratory have confirmed that scavenging active TGFβ1[76], inhibiting platelet aggregation[77], inhibiting thrombin function[27], mucosal immunomodulation[78], or inhibiting PAR_1 (unpublished data, 2005) all ameliorate various aspects of early and/or delayed radiation enteropathy. These studies are consistent with the notion that thrombin is a key link between downregulated TM and radiation-induced vascular and intestinal fibrosis.

Platelets

Thrombin, in addition to the properties described above, also has major effects on blood platelets. Platelets are the first cellular elements at the site of endothelial injury, where they initiate the hemostatic and inflammatory responses and contribute to the local cytokine milieu[79]. *In vivo* and *in vitro* studies have demonstrated that radiation enhances platelet adhesion[80] and platelet aggregation in the microvascular network[19,20]. The anti-platelet agent, acetylsalicylic acid (ASA, aspirin) may ameliorate certain aspects of intestinal and renal radiation toxicity[81-83].

Platelet adhesion, aggregation, and secretion are regulated by several mediators that are recognized by platelet surface receptors. Thrombin is a powerful platelet agonist and PARs mediate most of the actions of thrombin on platelet function[84]. Hence, PAR_1 activating peptide (PAR_1-AP) triggers complete platelet aggregation similar to the aggregation induced by thrombin. Adenosine diphosphate (ADP) is stored in platelet granules and is released in response to primary agonists, including thrombin. Thus, part of the response of platelets to thrombin is via autocrine and paracrine effects by secreted ADP[85]. In fact, some studies suggest that PAR_1-AP-induced aggregation may be entirely dependent on release of ADP[86]. ADP potentiates multiple platelet responses including the initiation of platelet aggregation (by receptor P2Y1) and the subsequent full aggregation and stabilization of platelet aggregates (by receptor P2Y12)[87,88]. Recent studies from our laboratory and others show that inhibition of ADP-induced platelet aggregation by clopidogrel or ticlopidine ameliorates early and delayed intestinal radiation toxicity[77,89].

Activated platelets directly elicit an inflammatory response by the production of free radicals and by the release of potent inflammatory mediators, such as, TGFβ, PAF, thromboxane, platelet derived growth factor, and IL1, which all contribute to chemoattraction and activation of inflammatory cells[79,90]. The ubiquitous proinflammatory, immunosuppressive, and fibrogenic growth factor, TGFβ, has been implicated in radiation injury such as skin, liver, heart, kidney, lung and intestine[76,91-93]. Platelets contain TGFβ in about 100-fold higher amounts than other types of cells or tissues. We have observed that TGFβ is expressed at significantly higher than normal levels after irradiation[94-96]. Moreover, our studies in radiation enteropathy were the first to demonstrate a mechanistic role for TGFβ in radiation-induced tissue toxicity[76].

Table 1 Potential pharmacological strategies for modulating post-radiation endothelial dysfunction to ameliorate development of radiation enteropathy and some of their respective limitations

Intervention	Major limitation
Platelet aggregation inhibitors	Narrow therapeutic window (bleeding)
Direct thrombin inhibitors	Narrow therapeutic window (bleeding)
Thrombin receptor blockers	Blocks only cellular thrombin effects
Recombinant thrombomodulin	Does not restore endothelial thrombomodulin
Activated protein C	Only partly blocks the effects of preformed thrombin
Statins	Non-specificity
Pentoxifylline	Non-specificity
Vitamin E	Non-specificity and variable efficacy

ENDOTHELIAL-ORIENTED APPROACHES TO MODULATE RADIATION ENTEROPATHY

As described in the previous sections of this review, radiation induces a plethora of changes in the microvascular endothelium. Some of these changes are transient, but may contribute to aspects of early radiation enteropathy. Other changes are sustained and may play direct roles in the pathogenesis of intestinal radiation fibrosis and in the mechanisms of chronicity and progression of injury. The postradiation shift in the thrombohemorrhagic balance toward procoagulation and the accompanying cellular effects that are the consequences of this shift represent particularly promising targets for intervention (Table 1).

Many of the conventional inhibitors of blood clotting have been tested in the attempt to ameliorate normal tissue radiation toxicity. The inconsistent results of these interventions are likely a result of the use of non-specific drugs with multiple actions, use of compounds with dose-limiting side-effects (primarily bleeding), and/or a too narrow focus on coagulation without appropriate consideration of the cellular effects of thrombin and the anti-inflammatory properties of the TM-protein C pathway. For example, while heparin is a highly effective anticoagulant, at therapeutic concentrations heparin reduces the affinity of thrombin for TM and the rate of protein C activation[97], and heparin administered at the time of irradiation actually exacerbates radiation-induced intestinal tissue injury[98]. The direct thrombin inhibitor, hirudin, ameliorates radiation enteropathy, but is less effective than an inhibitor of ADP-induced platelet aggregation, clopidogrel[27]. A possible explanation of these findings may be that thrombin inhibition also reduces thrombin-induced protein C activation and thereby the anti-inflammatory actions of APC. In contrast, inhibition of ADP-induced platelet aggregation targets processes downstream of thrombin and does not influence APC in the same manner. These observations are consistent with results from other studies showing that direct thrombin inhibition enhances leukocyte-endothelial cell interaction in endotoxin-induced sepsis[99] and, despite a favorable effect on collagen accumulation, does not affect inflammatory cell recruitment in bleomycin-induced lung injury[100].

Particularly attractive and presumably safe approaches to modulate radiation-induced endothelial dysfunction are to administer exogenous recombinant TM and APC, to restore endothelial cell TM, and/or to block the downstream effector of thrombin, PAR_1.

Recombinant human soluble TM (rhsTM) is composed of the active, extracellular domain of TM. rhsTM activates protein C[101], reduces thrombin generation[102], and prevents thrombosis *in vivo*[103-105]. The efficacy of rhsTM has been demonstrated in other situations associated with deficiency of endothelial TM, such as disseminated intravascular coagulation, experimental sepsis, and multiple system organ failure[42,105,106]. rhsTM also inhibits smooth muscle proliferation and vascular neointimal hyperplasia[107,108]. Although rhsTM has not yet been tested in the context of radiation toxicity, it is conceivable that rhsTM may be beneficial in normal tissue radiation toxicity. The objective would be to provide TM by the exogenous route for a limited period of time and thus allow TM to regenerate on the endothelial surface.

Synthetic TM mimics are compounds that change thrombin's substrate specificity in a fashion similar to TM and thus cause thrombin to activate protein C[109]. This is a new class of antithrombotic agents that exploits the powerful natural protein C anticoagulant pathway. This approach may be particularly appealing in the context of radiation enteropathy, because localized radiation does not cause protein C deficiency, but rather induces a decrease in local protein C activation due to lack of functional TM. However, while the TM mimics may have a superior therapeutic profile compared to direct thrombin inhibitors, TM mimics suitable for use *in vivo* are not yet available.

Replacement therapy with recombinant APC (rAPC) is another strategy that might allow endothelial function to recover and thus interrupt the vicious cycle that leads to radiation-induced organ dysfunction. APC possesses a number of properties that are different from those of conventional anticoagulants, including potent anti-inflammatory and cytoprotective activities[110-113]. Studies by others have shown that rAPC prevents the lethal effects of E. coli-associated sepsis in animal models and improves the outcome of patients with severe sepsis[114], and that short-term rAPC administration ameliorates lung fibrosis in bleomycin-induced lung injury[115]. Administration of rAPC during the early postradiation phase warrants investigation as an approach to mitigate radiation enteropathy development.

A particularly interesting approach to upregulate and/or restore endothelial TM is treatment with inhibitors of 3-hydroxy-3-methylglutaryl-coenzyme A (HMG-CoA) reductase, for example, the lipid-lowering statins. In 2003, we and a Japanese group demonstrated independently that statins, in addition to inhibiting the biosynthesis of cholesterol, strongly upregulate TM gene expression, protein levels, and function[116,117] and counteract the effects of TNFα on endothelial TM[116]. It was subsequently shown that statins attenuate radiation pneumonitis[118] and early radiation-induced intestinal toxicity[119]. Whether the radioprotective properties of statins are indeed attributable to their effect on TM expression or to other non-lipid-related statin effects, and whether the beneficial effect of statins in experimental models of normal tissue radiation toxicity can be translated to the clinical situation remains to be shown.

Pentoxifylline as monotherapy or in combination with tocopherol (vitamin E) is another approach that may ameliorate normal tissue radiation toxicity in some tissues by decreasing endothelial dysfunction and restoring endothelial TM. Pentoxifylline is a methylxanthine derivative with potent hemorrheologic properties. It improves blood fluidity by multiple effects such as increasing the deformability of red blood cells and leukocytes, preventing the aggregation of platelets, and decreasing plasma viscosity. It was originally developed for treatment of regional microcirculation disorders such as intermittent claudication and cerebrovascular disease. However, recent studies have shown that pentoxifylline possesses anti-inflammatory and immunomodulatory properties[120-122] and can be used as an adjuvant in the treatment of a diverse group of diseases, including sepsis and severe acute respiratory distress syndrome. Pentoxifylline increases endothelial TM expression and prevents hypoxic- and TNFα-induced reduction in TM expression[123,124]. Pentoxifylline also inhibits TF expression and counteracts activation of the coagulation cascade by endotoxin[125]. Clinical studies suggest that pentoxifylline may reverse radiation-induced chronic skin and subcutaneous tissue fibrosis[126]. Beneficial effects have also been observed in radiation-induced ulcer healing, as well as in radiation-induced toxicity in lung, intestine, uterine, breast, and jaw muscles[127-131]. Nevertheless, a number

of negative animal studies[132,133] and several inconclusive clinical reports highlight the need for further studies to define the benefits, indications, and mechanisms of action of pentoxifylline in radiation fibrosis.

Inhibition of PAR_1 may prove to be a particularly effective strategy to reduce radiation-induced normal tissue toxicity. Because PAR_1 antagonists are specific for the cellular actions of thrombin, it does not interfere with formation of the thrombin-TM complex and therefore does not reduce activation of protein C. Furthermore, since PAR_1 inhibitors do not interfere with fibrin generation, they will likely be associated with fewer bleeding complications than other anticoagulants. Several peptide and non-peptide (small molecule) PAR_1 antagonists are under development[134,135]. Some act on the extracellular portion of the receptors[134], whereas others act as intracellular inhibitors of signal transduction from receptors to G proteins[135]. Studies of PAR_1 inhibition as an approach to reduce normal tissue radiation toxicity are currently underway in our laboratory.

CONCLUSIONS

Normal tissue toxicity, including intestinal radiation toxicity, is the main dose-limiting factor during radiation therapy of cancer. Radiation enteropathy adversely impacts the therapeutic efficacy of radiation therapy, as well as the quality of life of long term cancer survivors. Clinical and preclinical evidence strongly suggests that endothelial dysfunction plays a critical role in the pathogenesis of early and delayed radiation enteropathy. Various endothelial-oriented pharmacological interventions are currently under development for the purpose of preventing or treating radiation enteropathy. Strategies aimed at restoring or preserving endothelial TM or blocking the thrombin receptor, PAR_1, hold particular promise, especially if interventions can be targeted to specific tissues or cellular compartments.

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