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Forum Review

Reactive Oxygen Species as Signaling Molecules in Cardiovascular Differentiation of Embryonic Stem Cells and Tumor-Induced Angiogenesis

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ABSTRACT

Besides the well known pathophysiological impact of oxidative stress in cardiovascular disease, reactive oxygen species (ROS) generated at low concentrations exert a role as signaling molecules that are involved in signal transduction cascades of numerous growth factor-, cytokine-, and hormone-mediated pathways, and regulate biological effects such as apoptosis, cell proliferation, and differentiation. Embryonic stem cells have the capacity to differentiate into the cardiovascular cell lineage. Furthermore, upon confrontation culture with tumor tissue, they form blood vessel-like structures that induce tumor-induced angiogenesis within tumor tissues. The role of ROS in cardiovascular differentiation of embryonic stem cells appears to be antagonistic. Whereas continuous exposure to ROS results in inhibition of cardiomyogenesis and vasculogenesis, pulse-chase exposure to low-level ROS enhances differentiation toward the cardiomyogenic as well as vascular cell lineage. This review summarizes the current knowledge of ROS-induced cardiovascular differentiation of embryonic stem cells as well as the role of ROS in tumor-induced angiogenesis. *Antioxid. Redox Signal.* 7, 1423–1434.

INTRODUCTION

IT IS WELL ACCEPTED that reactive oxygen species (ROS) are causing agents in the pathogenesis of various cardiovascular diseases, including hypertension, artherosclerosis, restenosis, cardiac hypertrophy, and heart failure (29). Additionally, oxidative stress arising from increased production of ROS has been implicated in the development of diseases like cancer and neurological disorders such as Cruetzfeldt–Jacob disease (14) and Alzheimer disease (40). However, besides their pathophysiological roles, ROS generated at low concentrations exert a role as signaling molecules that are involved in signal transduction cascades of numerous growth factor-, cytokine-, and hormone-mediated pathways, which regulate biological effects such as apoptosis, cell proliferation, and differentiation (77, 90). Embryonic stem cells have the capacity to differentiate into the cardiovascular cell lineage and form blood vessel-like structures that mediate tumor-induced angiogenesis within tumor tissues. It has been demonstrated that embryoid bodies grown from embryonic stem cells actively generated ROS presumably through the activity of ROS-generating NAD(P)H oxidase (87). These ROS could be utilized within signaling cascades that result in the transcription of genes directing differentiation toward the cardiomyogenic as well as endothelial cell lineage. A role of ROS in embryogenesis has been discussed for more than 10 years. Based on evidence from a variety of sources, it was suggested that oxygen free radicals may exert a decisive role in cellular differentiation (97). It was postulated that differentiated cells have a relatively more prooxidizing or less reducing intracellular environment than the undifferentiated or dedifferentiated cells. Changes in the redox balance during differentia-

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tion appear to be due to an increase in the rate of superoxide ($O_2^{-\cdot}$) generation. Differentiated cells, in general, exhibit higher rates of cyanide-resistant respiration, cyanide-insensitive superoxide dismutase activity, and peroxide concentration and lower levels of reduced glutathione as compared with undifferentiated cells (97). According to the free radical theory of development established by Allen and Balin (4), metabolic gradients exist in developing organisms and are believed to influence development. The effects of these gradients on development result from differential oxygen supplies to tissues and may cause the formation of metabolically generated oxidant gradients that direct the initiation of certain developmental events.

NAD(P)H OXIDASE IN ENDOTHELIAL AND SMOOTH MUSCLE CELLS

In the past decade, it has become evident that NAD(P)H oxidases are major sources of ROS in vascular as well as smooth muscle cells. Initially, structure and function of NAD(P)H oxidases have been investigated in detail in neutrophils that utilize high levels of ROS to kill invading bacteria and other microorganisms. The neutrophil NAD(P)H oxidase comprises two membrane-associated subunits, $p22^{phox}$ and $gp91^{phox}$, which form cytochrome b558, and the cytoplasmic subunits $p47^{phox}$, $p40^{phox}$, $p67^{phox}$, and the small GTP-binding protein Rac. Upon stimulation of phagocytic cells, the cytosolic subunits translocate to cytochrome b558 at the membrane, which results in activation and the oxidative burst.

In contrast to neutrophils, NAD(P)H oxidases in nonphagocytic cells produce ROS at low levels. This occurs when cells are stimulated with cytokines, growth factors, and hormones, *e.g.,* interleukin (IL)-1β (109), IL-6 (102), IL-3 (86), tumor necrosis factor-α (81), angiotensin-II (Ang-II) (112, 113, 129), platelet-derived growth factor (PDGF) (101), nerve growth factor (103), transforming growth factor-β1 (TGF-β1) (106), granulocyte-macrophage colony-stimulating factor (86), vascular endothelial growth factor (VEGF) (26), fibroblast growth factor-2 (61), and cardiotrophin-1 (CT-1) (91). These observations led to the conclusion that the initiation and/or functioning of a variety of signaling cascades rely on ROS as signaling molecules that may be active on different levels of the signaling cascade. ROS are ideally suited as signaling molecules because they are rapidly generated, highly diffusible, and have a short half-life.

In recent years, a family of $gp91^{phox}$-like nonphagocytic NAD(P)H oxidase proteins have been described and have been named NOX proteins. Currently, this family comprises NOX1, NOX2, NOX3, NOX4, and NOX5 (18). Additionally, two further proteins of distinct NOX homology, named DUOX1 and DUOX2, display NOX-homologous regions and peroxidase activity (53). It has been shown that endothelial cells express NOX1, NOX2, NOX4, and NOX5. Vascular smooth muscle cells express NOX1, NOX4, and NOX5 (57). Recently, two proteins with homology to $p47^{phox}$ and $p67^{phox}$ have been characterized and named NOX organizer 1 and NOX activator 1 because they regulate NOX1 activity (8, 33, 105). ROS generated from NAD(P)H oxidases expressed in endothelial as well as smooth muscle cells have been demonstrated to be involved in the pathophysiology of several cardiovascular disorders, including artherosclerosis, hypertension, heart failure, and diabetic vasculopathy.

NAD(P)H OXIDASE IN THE HEART

Whereas the expression of NAD(P)H oxidase in endothelial as well as smooth muscle cells has been investigated in detail, the sources of ROS in cardiac cells are not well described. Most recent studies focused on the crucial role of oxidative stress as causative for ventricular dysfunction that results in chronic congestive heart failure (117). Increased myocardial NADPH oxidase activity was found in human heart failure. The NADPH oxidase subunits $p22^{phox}$, $gp91^{phox}$, $p67^{phox}$, and $p47^{phox}$ were all expressed at mRNA and protein level in cardiomyocytes of both nonfailing and failing hearts (39). Recently, expression of NOX2 has been demonstrated in human cardiomyocytes and was shown to be up-regulated during acute myocardial infarction (52). Excess generation of ROS may alter the activity and expression of proteins involved in excitation–contraction coupling, such as L-type calcium channels (19), ion exchangers (44), sarcoplasmic reticulum calcium release channels (23, 104), and myofilament proteins (62). Activation of NAD(P)H oxidase during progression of cardiac hypertrophy to failure has been previously evidenced (58). It has been shown that ROS mediate α-adrenergic receptor-stimulated hypertrophy in adult rat ventricular myocytes (6, 124). Furthermore, β-adrenergic receptor-stimulated apoptosis in cardiac myocytes has been shown to be mediated by ROS/c-Jun NH_2-terminal kinase (JNK)-dependent activation of the mitochondrial pathway (82). During recent years, pivotal interest has been attributed to ROS in Ang-II signaling pathways. Ang-II is responsible for vascular remodeling after myocardial infarction (78) and may be an important mediator in tissue fibrosis (108). Bendall *et al.* (12) provided evidence that the hypertrophic response of the myocardium to subpressor doses of Ang-II is regulated by the myocardial NAD(P)H oxidase because this phenomenon was completely prevented in $gp91^{phox-/-}$ mice. Recently, it was demonstrated that ROS are involved in Ang-II-induced proliferation and endothelin-1 (ET-1) gene expression, which implies that the combination of AT(I) and ET(A) receptor antagonists plus antioxidants may be beneficial in preventing the formation of excessive cardiac fibrosis (22). However, it may be possible that Ang-II may contribute to cardiac differentiation in nonpathologic states. In this respect, it has been shown that Ang-II added to cultures of whole rat embryos resulted in increased ventricular growth and myocyte hypertrophy (79). This effect may be related to the generation of ROS because NADPH oxidase-derived superoxide anions have been shown to mediate cell growth in cultured neonatal cardiac cells (69). The role of Ang-II in stem cell differentiation has not been investigated yet. However, the existence of a bone marrow renin–angiotensin system has been recently evidenced, which suggests a possible role for Ang-II in cell differentiation processes of different types of stem cells (100).

REGULATION OF CARDIOMYOGENESIS OF EMBRYONIC STEM CELLS BY REDOX CHANGES

Embryonic stem cells are pluripotent and have been shown to differentiate into cardiomyocytes. In mouse embryonic stem cells, cardiac development within embryoid bodies occurs as early as 7 days after formation of the aggregates, which correlates well with the murine embryo where the first beating is seen on embryonic day 8.5–9.5 (*i.e.,* 8.5–9.5 days post coitum) (3). It was found that the earliest detectable cardiomyocytes (stage 0) were not beating, but expressed already voltage-dependent L-type Ca^{2+} channels at low density (3). During the further developmental stages (stage 1–4), spontaneous contracting activity occurs and the increasing number of different ion channels causes a diversification of cardiac phenotypes, finally leading to the known specialized cardiomyocytes as likewise found in the neonatal heart, *i.e.,* ventricular-like, atrial-like, and sinus-nodal-like, as well as Purkinje-like cardiomyocytes (63, 64). The molecular signaling pathways that direct embryonic stem cells toward the cardiomyogenic cell lineage are vastly unknown. However, increasing scientific evidence suggests that cardiomyogenesis in embryonic stem cells may be facilitated by nitric oxide (NO) as well as by ROS. Prominent expression of both inducible nitric oxide synthase (iNOS) and endothelial NOS (eNOS) proteins has been observed in the heart during embryonic development starting on day 0.5 (16). This prominent expression abates before birth, suggesting that a period of NO exposure is required for normal development. eNOS knockout mice display severe congenital heart malformations, such as bicuspid aortic valves and atrial and ventricular septal defects (27, 55). In mouse embryonic stem cells, NOS inhibitors have been shown to arrest differentiation toward a cardiac phenotype (15). This study demonstrated that NO donors restored cardiac differentiation (15). Recently, it was shown that NO facilitates cardiomyogenesis in mouse embryonic stem cells. NO treatment as well as overexpression of iNOS increased both the number and the size of beating foci in embryoid body outgrowths. Interestingly, it was observed that NO not only induced differentiation, but also initiated apoptosis in undifferentiated cells, which led to the suggestion that NO may influence cardiac differentiation by both inducing a switch toward a cardiac phenotype and inducing apoptosis in cells not committed to cardiac differentiation (48).

Besides NO, cardiomyogenesis has likewise been shown to be stimulated by ROS. During embryoid body differentiation, embryoid bodies displayed significant endogenous production of ROS, which was accompanied by the expression of the NADPH oxidase subunit $p67^{phox}$ (87) (Fig. 1). The activity of NADPH oxidase was apparently regulated by phosphatidylinositol 3-kinase (PI3-kinase) because inhibitors of PI3-kinase down-regulated ROS generation in embryoid bodies (88). In Caco-2, HEK293T, and Cos-7 cells, it has been recently shown that ROS production in growth factor-stimulated cells is mediated by the sequential activation of PI3-kinase, β Pix, and Rac1, which then binds to Nox1 to stimulate its NADPH oxidase activity (73). Recently, it was evidenced that Akt mediates PI3-kinase-dependent $p47^{phox}$ phosphorylation, which contributes to respiratory burst activity in human neutrophils (21). A requirement of PI3-kinase activity for cardiomyogenesis of embryonic stem cells was previously shown because differentiation of beating foci of cardiac cells was severely inhibited in the presence of PI3-kinase inhibitors (50). Enhancement of ROS production was induced by pulse-chase electrical field treatment on day 4 of cell culture prior to differentiation of cardiac cell clusters. This treatment significantly increased the production of ROS immediately after electrical field treatment and over a time period of > 48 h (92). Following 24 h of electrical field treatment, up-regulation of the NADPH oxidase subunits $p22^{phox}$, $p47^{phox}$, $p67^{phox}$, and $gp91^{phox}$ was observed, indicating a feed-forward regulation of NADPH oxidase expression by ROS (92). Electrical field-treated embryoid bodies displayed increased numbers of spontaneously beating foci of cardiomyocytes as well as an increased size of beating foci and activated the redox-regulated transcription factor nuclear factor-κB (NF-κB) (87). The observed effects were abolished by free radical scavengers as well as by the NF-κB inhibitor *N*-tosyl-L-phenylalanine chloromethyl ketone. NF-κB functions as a key regulator of cardiac gene expression programs downstream of multiple signal transduction cascades in a variety of physiological and pathophysiological states (47). Previously, it was shown that the principal NF-κB subunits p65, p50, IκB-α, and IκB-β are present throughout development, suggesting

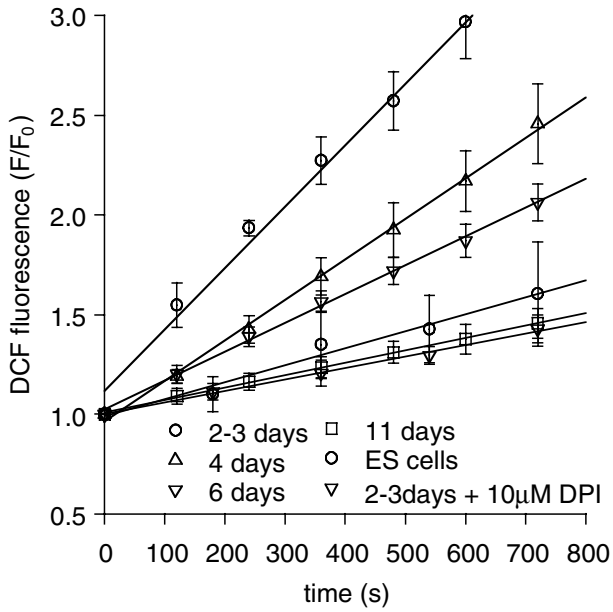

FIG. 1. Endogenous generation of ROS in embryonic stem cells and embryoid bodies during different developmental stages. The graph shows the time course of 2′,7′-dichlorodihydrofluorescein (H_2DCF) oxidation in undifferentiated embryonic stem (ES) cells, in 2–3-, 4-, 6-, and 11-day-old embryoid bodies and in 2–3-day-old embryoid bodies in the presence of 10 μ*M* diphenyleneiodonium (DPI), which is a nonspecific inhibitor of NAD(P)H oxidase. Embryoid bodies were incubated with 20 μ*M* H_2DCF diacetate, and the oxidation of nonfluorescent H_2DCF to fluorescent 2′,7′-dichlorofluorescein (DCF) was monitored. From (88).

that this transcription complex may participate in myocardial gene regulation throughout development and in the adult (71). NF-κB activation has been recently demonstrated to be required for the development of cardiac hypertrophy *in vivo* (59). In comparing the pathological state of cardiac hypertrophy with early embryonic growth and development of the primitive heart, important and informative aspects of mechanisms that underlie activation of the gene expression pattern become apparent. In both cases, the muscle phenotypes share the expression of a fetal gene expression program, raising the question whether the same genetic mechanism is being called upon by signals associated with the onsets of cardiogenesis and myocardial hypertrophy (35).

The effect of electrical field treatment of embryoid bodies could be mimicked by exogenous addition of hydrogen peroxide. Concentrations of hydrogen peroxide as low as 10–100 n*M*, applied in a pulse-chase protocol on day 4 of cell culture, significantly enhanced cardiomyogenesis in embryoid bodies, whereas higher concentrations exceeding 1 μ*M* exhibited an inhibitory effect (87). Under these conditions, oxidative stress is induced that has previously been shown to selectively inhibit muscle gene expression in cardiac cells (110). Consequently, a more recent study demonstrated that cardiomyogenic differentiation of embryonic stem cells is abrogated by treatment with the anticonvulsant valproic acid, which induced oxidative stress in embryoid bodies (68). *In vivo* valproic acid exerts teratogenic properties and has been demonstrated to cause neural tube defects and malformations of the heart (107). Oxidative stress may likewise have been the causative for the reduction of cardiomyogenesis and down-regulation of the cardiac-specific transcription factor MEF2C observed in embryonic stem cells overexpressing a constitutively active Rac (RacV12), which was recently demonstrated in an elegant and detailed study by Pucéat *et al.* (80). Rac1 and Rac2 are known to bind to $p67^{phox}$, thereby activating the NAD(P)H oxidase (70). Continuous addition of hydrogen peroxide from day 0 to day 7 of cell culture inhibited cardiomyogenesis. Incubation of RacV12 embryonic stem cells with the free radical scavenger catalase restored cardiomyogenesis, which clearly indicated that continuous elevation of intracellular ROS is deleterious for cardiomyogenesis. Interestingly, this study demonstrated that ventricular-specific, late expression of RacV12 under control of the ventricular myosin light chain 2 promoter significantly improved or accelerated the process of cardiac differentiation. Hence, when expressed only in ventricular cells and at a stage of differentiation when cardiac transcription factors have already reached maximal level of expression, Rac GTPase activity may be required for terminal differentiation and proliferation of cardiac precursors. In contrast, early and ubiquitous overexpression of Rac resulting in overall oxidative stress may prevent differentiation of the cardioblast to terminally differentiated cardiac cells and may regulate embryonic apoptosis. Rac-induced ROS have been previously shown to be critical for physiological apoptosis occurring during early embryonic organogenesis and regulating heart formation (76).

A previous study of Behfar *et al.* (9) demonstrated that members of the TGF-β superfamily, *i.e.,* TGF-β1 and bone morphogenic protein-2, up-regulated mRNA of mesodermal (brachyury) and cardiac-specific (Nkx2.5, MEF2C) transcription factors, and increased the potential for cardiac differentiation. Extracellular signal-regulated kinase (ERK) and Rac contribute to the effects of TGF-β1 on gene expression (67). TGF-β1 has been previously shown to increase intracellular ROS in hepatocytes (38), glomerular mesangial cells (46), and alveolar epithelial cells (45). A comparable mechanism of ROS-based stimulation of cardiomyogenesis could underly PDGF-BB treatment of embryonic stem cells, which was recently reported (84). PDGF is well known to increase ROS in a variety of cells, including lens epithelial cells (20), vascular smooth muscle cells (51, 122), and human skin fibroblasts (13), and has been demonstrated to involve Rac activation in its signal transduction cascade (24, 85). Besides growth factors, cardiomyogenesis in embryonic stem cells has been demonstrated to be stimulated by the cytokine CT-1 (91). CT-1 belongs to the pleiotrophic family of cytokines that includes IL-6, IL-11, leukemia inhibitory factor (LIF), ciliary neurotrophic factor, and oncostatin M, which activate downstream signal transduction cascades via gp130-dependent pathways (37). During mouse embryogenesis (days 8.5–11.5) CT-1 is preferentially expressed in the heart, whereas expression is less restricted in later stages of embryogenesis and has been observed in the adult in several tissues, including skeletal muscle, lungs, brain, and liver, suggesting broader biological effects of CT-1 exceeding its action in the heart (96). CT-1 has been demonstrated to induce proliferation in embryonic, and to promote survival of neonatal cardiomyocytes (96). In adult cells, CT-1 induces hypertrophy (75) and protects cardiac cells from injury (17, 60). It was shown that CT-1 raised intracellular ROS in cardiac cells isolated from embryonic stem cells and promoted cell proliferation. The downstream signaling cascade activated by CT-1 in embryonic stem cells, *i.e.,* activation of the Jak/STAT, ERK1,2, and NF-κB, required ROS for activation. The Jak/STAT (74, 94), the ERK (36, 72), and the NF-κB (25, 95) signaling cascades have been previously demonstrated to be regulated by intracellular ROS in a number of cell types, raising the possibility that CT-1 exerts its biological effects via elevation of ROS, which act as signaling molecules in CT-1-induced signal transduction cascades (Fig. 2).

REGULATION OF ANGIOGENESIS BY ROS

The implication of endothelial cell ROS generation in the regulation of vascular function and vascular pathophysiology has been extensively studied in recent years. Endothelial NAD(P)H oxidase activity is increased by mechanical forces such as oscillatory shear stress (41), hypoxia–reoxygenation (49, 93, 99), flow cessation (65), and membrane depolarization (5, 98). It has been known for a long time that angiogenesis is stimulated by hypoxia (125). VEGF expression is under the control of hypoxia-inducible factor-1α (HIF-1α). Under normoxia, the HIF-1α subunit is rapidly degraded via the von Hippel–Lindau tumor suppressor gene product (pVHL)-mediated ubiquitin-proteasome pathway. The association of pVHL and HIF-1α under normoxic conditions is triggered by the hydroxylation of prolines and the acetylation of lysine within a polypeptide segment known as the oxygen-de-

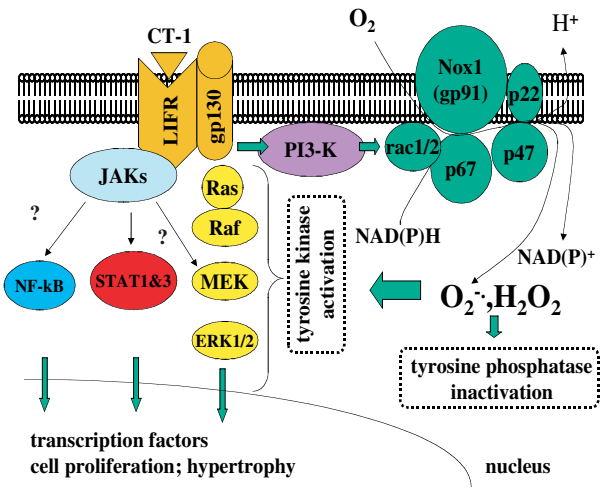

FIG. 2. Schematic diagram of the involvement of ROS in CT-1-induced signaling cascades. Upon stimulation of the gp130/LIF receptor heterodimer, NADPH oxidase [consisting of the subunits Nox-1 (gp91), p22, p47, p67, and rac1/2] is activated through PI3-kinase. The ROS generated by NADPH oxidase interfere with Jak/STAT, ERK1/2, and NF-κB signaling pathways and mediate activation. The activation of STAT-3 and NF-κB, but not Jak-2 phosphorylation, is critically dependent on the ERK signaling pathway. From (91).

pendent degradation domain. On the contrary, in the hypoxia condition, the HIF-1α subunit becomes stable and interacts with coactivators such as p300/CBP to modulate its transcriptional activity (54). Recently, HIF-1α and consequently VEGF expression have been shown to be up-regulated in response to ROS exposure, underscoring a role for ROS in vascular growth and differentiation (10, 32). Furthermore, ROS have been implicated in the regulation of endothelial cell proliferation, migration, and organization into tubular network structures, which are critical steps in angiogenesis. In proliferation assays, growth factor- or serum-induced DNA synthesis in three different types of human endothelial cells was abrogated by inhibitors of NADPH oxidase, but not by inhibitors of xanthine oxidase or NOS. Moreover, VEGF-induced migration of human endothelial cells was suppressed in the presence of NADPH oxidase inhibitors (1). Ushio-Fukai *et al.* (114) showed that VEGF-induced endothelial cell proliferation, migration, and angiogenesis were inhibited by dominant-negative Rac1 or antisense $gp91^{phox}$ oligonucleotides, which reduced VEGF-induced superoxide generation. During shear stress-induced migration, endothelial cells reorient by a two-step process involving Rho-induced depolarization, followed by Rho/Rac-mediated polarization and migration in the direction of flow (123). In an endothelial monolayer-wounding assay, ROS generation in response to the loss of endothelial confluence was required for actin cytoskeleton reorganization, which is a prerequisite for endothelial cell regeneration and proliferation (66). Recently, IQGAP1, a novel scaffolding protein, was discovered that controls cellular motility and morphogenesis by interacting directly with cytoskeletal, cell adhesion, and small G proteins, including Rac1. It was shown that IQGAP1 functions as a VEGF receptor2-associated scaffold protein to organize ROS-dependent VEGF signaling, thereby promoting endothelial cell migration and proliferation, which may contribute to repair and maintenance of the functional integrity of established blood vessels (126). VEGF is uniquely coupled to manganese superoxide dismutase expression through growth factor-specific ROS-sensitive positive (protein kinase C–NF-κB) and negative (PI3-kinase–Akt–forkhead) signaling pathways to avoid oxidative stress arising during growth factor-induced ROS generation (2).

The role of ROS for vascular differentiation and angiogenesis of stem cells is poorly described. Recently, it was reported that migration of human hematopoietic progenitor cells across bone marrow endothelium is regulated by vascular endothelial (VE) cadherin. Loss of VE cadherin-mediated endothelial cell–cell adhesion increased the permeability of monolayers of human bone marrow endothelial cells (HBMECs) and stimulated the transendothelial migration of $CD34^+$ cells in response to stromal cell-derived factor-1α. Vascular cell adhesion molecule-1 (VCAM-1)-mediated gap formation in HBMECs was accompanied by and dependent on the production of ROS, which suggested that modulation of VE cadherin function directly affected the efficiency of transendothelial migration of $CD34^+$ cells. Activation of intercellular adhesion molecule-1 and, in particular, VCAM-1 apparently played an important role in this process through reorganization of the endothelial actin cytoskeleton and by modulating the integrity of the bone marrow endothelium through the production of ROS (115).

REGULATION OF VASCULOGENESIS AND ANGIOGENESIS OF EMBRYONIC STEM CELLS BY REDOX CHANGES

The adult vasculature results from a network of vessels that is originally derived in the embryo by vasculogenesis, a process whereby vessels are formed *de novo* from endothelial cell precursors, known as angioblasts. During vasculogenesis, angioblasts proliferate and come together to form an initial network of vessels, also known as the primary capillary plexus. Sprouting and branching of new vessels from the preexisting vessels in the process of angiogenesis remodel the capillary plexus. Normal angiogenesis, a well balanced process, is important in the embryo to promote the primary vascular tree as well as an adequate vasculature from developing organs. Vasculogenesis, the *in situ* assembly of capillaries from undifferentiated endothelial precursor cells, and angiogenesis, the sprouting of capillaries from preexisting blood vessels, have been extensively studied in embryonic stem cells of mouse (28, 116) and human (56) origin. It has been discussed that the vasculogenic potential of embryonic stem cells could be specifically of use in tissue engineering for the induction of tissue vascularization (56). It has been conclusively demonstrated that embryonic stem cell-derived embryoid bodies represent a suitable *in vitro* model to study molecular events involved in vascular development. Embryonic stem cells differentiate *in vitro* to endothelial cells through successive maturation steps with sequential expression of cell

lineage-specific markers: platelet endothelial cell adhesion molecule (PECAM), Flk-1, tie-1, tie-2, VE cadherin, MECA-32, and MEC-14.7 (116). These endothelial cells differentiated from embryonic stem cells form functional capillary structures that facilitate diffusion and dissipate oxygen gradients within the tissue. We have recently introduced the embryoid body as a model system for *in vitro* testing of antiangiogenic agents (118).

The redox control of angiogenesis requires a tightly regulated balance. Accumulating evidence suggests that cardiovascular diseases are associated with increased oxidative stress in blood vessels. ROS such as superoxide and hydrogen peroxide cause blood vessels to thicken, produce inflammation in the vessel wall, and thus are regarded as "risk factors" for vascular disease (111). It has been recently shown that the antiangiogenic effect of the sedative thalidomide in embryonic stem cell-derived embryoid bodies is mediated by chronic elevation of highly reactive hydroxyl radicals (89) because vasculogenesis/angiogenesis could be restored by coadministration of hydroxyl radical scavengers. A comparable mechanism of action may also prevail in the antiangiogenic action of the antimalaria agent artemisinin (121). The mode of action of artemisinin on *Plasmodium falciparum* has been associated with the generation of oxidative stress by this compound because the intraerythrocytic activation of the drug peroxide bond by iron(II)-heme produced during hemoglobin degradation should generate ROS (83). Recently, it has been reported that ROS derived from NAD(P)H oxidase are critically important for VEGF signaling *in vitro* and angiogenesis *in vivo* (126). Comparable signaling cascades may also prevail in embryonic stem cells. In this respect, it has been recently shown that electrical field-induced elevation of ROS and induction of NAD(P)H oxidase expression significantly stimulated capillary structure formation in embryonic stem cell-derived embryoid bodies and elevated HIF-1α as well as VEGF in a redox-sensitive manner (92). Electrical field treatment resulted in activation of ERK1,2, p38, and JNK. Pretreatment with the JNK inhibitor SP600125 resulted in a significant decrease in capillary areas under control conditions as well as under conditions of electrical field treatment, whereas the p38 inhibitor SB203580 was without effects. By contrast, the ERK1,2 antagonist UO126 inhibited electrical field-induced angiogenesis, whereas angiogenesis under control conditions was unimpaired. The increase in capillary areas and VEGF expression as well as activation of JNK and ERK1,2 was significantly inhibited in the presence of the free radical scavenger vitamin E, underscoring the role of ROS in electrical field-induced angiogenesis of embryonic stem cells.

ROS IN TUMOR-INDUCED ANGIOGENESIS MEDIATED BY EMBRYONIC STEM CELLS

It is a well known feature that tumors cannot grow beyond a size of a few millimeters without blood supply from the host tissue, a process called tumor-induced angiogenesis. This observation has led to the development of the antiangiogenic therapy by Folkman and colleagues (30, 31) that is based on the assumption that inhibition of tumor-induced angiogenesis would deprive the growing tumor from nutrients and oxygen supplied by the host circulation and, consequently, would retard or even abolish tumor growth. During recent years, a variety of pro- and antiangiogenic agents have been found, many of them now being tested in clinical trials (43). However, the molecular mechanisms of action of most compounds that are active in inhibiting angiogenesis are currently unknown. It was suggested that ROS are mediating the angiogenic switch, thereby increasing the vascularity of tumors and inducing molecular markers of angiogenesis (7). Recently, it was shown that JunD reduces tumor angiogenesis by protecting cells from oxidative stress (34). Using junD-deficient cells, it was demonstrated that JunD regulates genes involved in antioxidant defense, hydrogen peroxide production, and angiogenesis. The accumulation of hydrogen peroxide in *junD*$^{-/-}$ cells decreases the availability of iron (II) and reduces the activity of HIF prolyl hydroxylases that target HIF-1α for degradation. Subsequently, HIF-α proteins accumulate and enhance the transcription of *VEGF-A*. Furthermore, ROS have been demonstrated to be involved in the progression of tumor-induced angiogenesis because matrix metalloproteinase (MMP) expression is regulated by the intracellular redox state (42, 127). The presence of MMPs is essential for endothelial cell ingression into the tumor tissue because they degrade the extracellular matrix of the tumor cells and free the way for migrating endothelial cells (128). Embryonic stem cells are well suited to study tumor-induced angiogenesis. We have recently introduced a novel confrontation culture model based on multicellular tumor spheroids and embryonic stem cell-derived embryoid bodies (119). It was shown that within days of confrontation culture endothelial cells differentiating from embryonic stem cells invaded the tumor tissue, which resulted in tumor vascularization and consequently increased tumor growth. Interestingly, confrontation culture resulted in increased ROS generation in both the embryoid body and the tumor spheroid, suggesting that tumor-induced angiogenesis requires the presence of ROS for endothelial cell invasion. In parallel with the observed vascularization of tumor spheroids, up-regulation of MMP1, MMP2, and MMP9 was observed, which was abolished in the presence of free radical scavengers (120). Free radical scavengers likewise abolished tumor vascularization (Fig. 3), which suggests that redox-regulated MMP expression is a prerequisite for vascular growth within the tumor tissue with subsequent tumor expansion.

CONCLUSIONS AND OUTLOOK

Emerging evidence suggests that ROS not only are causatives for cardiovascular disease, but are involved in a variety of growth factor- and cytokine-mediated signaling cascades that regulate cell growth, differentiation, and apoptosis. The signaling events resulting in the cell lineage-specific differentiation of embryonic stem cells are currently nearly unknown. However, cardiac as well as vascular differentiation of embryonic stem cells has been demonstrated to be facilitated either by pulse-chase increase in ROS production or by growth factors that utilize ROS within their signaling cascades. ROS may play a similar role in adult stem cells.

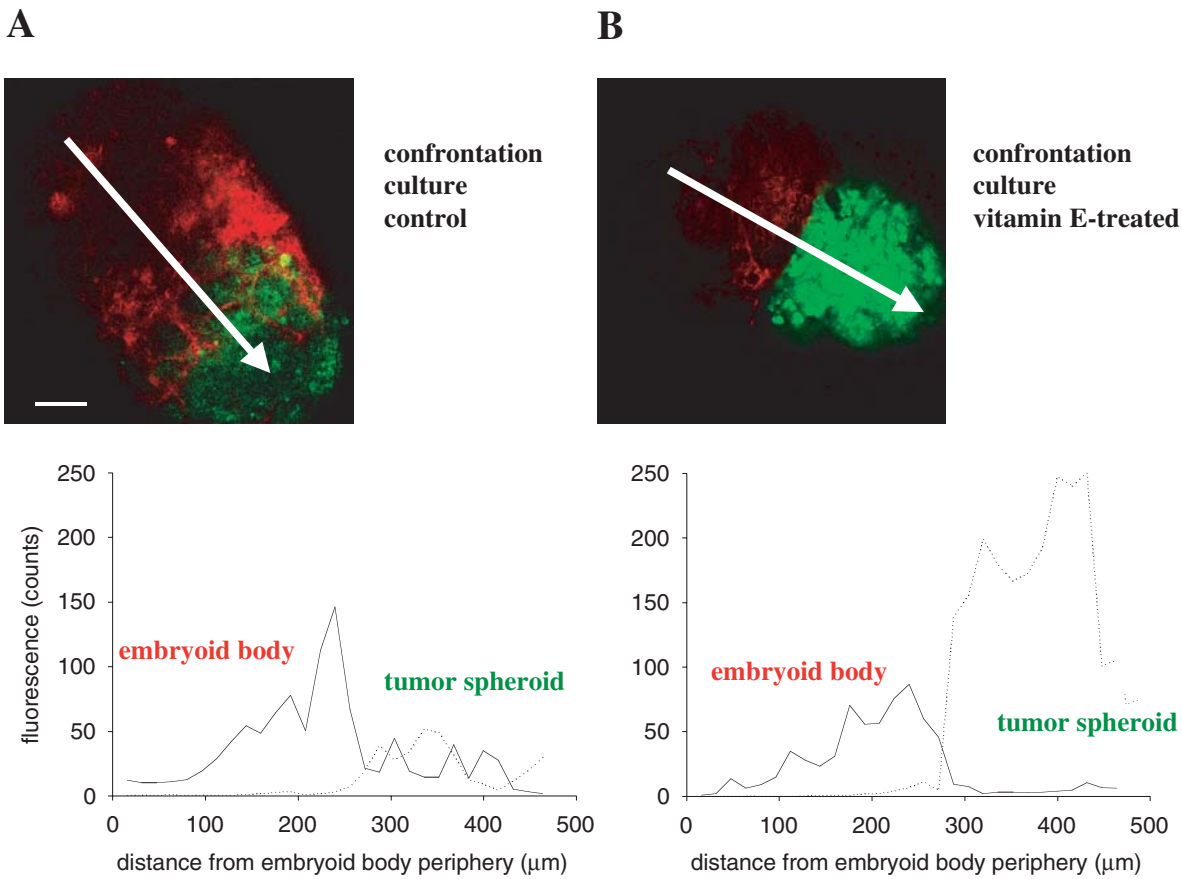

FIG. 3. Effect of the free radical scavenger vitamin E on tumor-induced angiogenesis in confrontation cultures consisting of embryoid bodies and multicellular tumor spheroids (5 days of confrontation culture). Endothelial cells were visualized by anti-PECAM-1 immunohistochemistry (red color). The tissue of multicellular tumor spheroids was visualized by labeling with the long-term cell tracker dye 5-chloromethylfluorescein diacetate (CMFDA) (green color). (**Upper panels**) Representative confrontation cultures that either remained untreated (**A**) or were treated for 5 days with vitamin E (100 μ*M*) (**B**). The bar represents 100 μm. It is clearly evident that in the control sample PECAM-1-positive capillary-like structures invaded the tumor spheroid, whereas no invasion occurred in the vitamin E-treated sample. Invasion of PECAM-1-positive (CMF-negative) endothelial cells into the tumor tissue resulted in a decline of CMF fluorescence in tumor spheroids. (**Lower panels**) Histograms of PECAM-1 fluorescence (solid line) and CMF fluorescence (dotted line) along the arrow in the images. Data are presented as fluorescence counts in relation to the distance from the embryoid body periphery. From (121).

Resident cardiac progenitor cells have been recently identified (11); however, the molecular mechanisms resulting in their commitment to the cardiomyogenic and vascular cell lineage remain to be identified. One (or the only) stimulus for commitment of resident stem cells is tissue injury that is frequently associated with inflammation and generation of ROS. Hence, ROS generated during tissue injury may activate resident stem cells or attract circulating stem cells to the site of inflammation. In adult amitotic cardiac cells, mechanical as well as biochemical stimuli induce ROS and elicit hypertrophic cell growth that is accompanied by reestablishment of a fetal gene program. In this respect, it sounds reasonable that comparable signaling events will facilitate cardiac cell differentiation and/or proliferation of stem cells. Further scientific efforts undertaken to unravel the mechanisms of cardiac fetal gene program activation will therefore not only give clues to the understanding of development of cardiac hypertrophy and heart failure, but will also add to the understanding of the molecular pathways resulting in cardiovascular differentiation of embryonic stem cells.

ABBREVIATIONS

Ang-II, angiotensin-II; CT-1, cardiotrophin-1; eNOS, endothelial nitric oxide synthase; ERK1,2, extracellular signal-regulated kinase 1,2; ET, endothelin; HBMEC, human bone marrow endothelial cell; HIF-1α, hypoxia-inducible factor-1α; IL, interleukin; iNOS, inducible nitric oxide synthase; JNK, c-Jun NH_2-terminal kinase; LIF, leukemia inhibitory factor; MMP, matrix metalloproteinase; NF-κB, nuclear factor-κB; NO, nitric oxide; NOS, nitric oxide synthase; PDGF, platelet-derived growth factor; PECAM-1, platelet endothelial cell adhesion molecule-1; PI3-kinase, phosphatidylinositol 3-kinase; pVHL, von Hippel–Lindau; ROS, reactive oxygen species; TGF-β1, transforming growth factor-β1;

VCAM-1, vascular cell adhesion molecule-1; VE, vascular endothelial; VEGF, vascular endothelial growth factor.

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