Effect of N-Acetyl Cysteine and α-Linolenic Acid on Sulfur Mustard Caused Impairment of In Vitro Endothelial Tube Formation

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Sulfur mustard (SM), an alkylating chemical warfare agent, leads to tissue damage, including inflammation, blister formation, and impaired wound healing. Especially wound healing is of concern because after SM exposure, wound healing is prolonged. In this study, we focused on the effect of SM (30 and 100 μM) on endothelial tube formation, apoptosis, and proliferation in mouse embryoid bodies (EBs), which provide an appropriate model for investigating vasculogenesis and angiogenesis. EBs were exposed to SM for 30 min on day 0, 3, or 6 of EBs’ growth, were allowed to grow until day 7, then fixed, and immunostained (PECAM-1, Ki67, and activated caspase-3). SM significantly decreased endothelial tube formation compared with unexposed EBs. Additionally, we observed a significant increase of apoptosis. As the formation of reactive oxygen species (ROS) is discussed to be involved in the pathophysiology of SM toxicity, we evaluated the effect of ROS scavengers (α-linolenic acid [ALA] and N-acetyl cysteine [NAC]) in the same experimental setup. Temporary effects of both scavengers could be detected, in particular NAC seemed to have temporary significant positive effects on endothelial tube formation in 100 μM SM–exposed EBs. ALA augmented proliferation when administered after 30 μM SM exposure on day 3, whereas NAC treatment on day 0 decreased apoptosis induced by 100 μM SM. Taken together, our findings pointed to a negative effect of SM on vascularization and endothelial tube formation. ROS scavengers NAC and ALA showed temporary, but not long-lasting, rescuing effects regarding endothelial tube formation after SM exposure.

Key Words: sulfur mustard; N-acetyl cysteine; α-linolenic acid; endothelial progenitor cells; embryoid body; wound healing.

Sulfur mustard (SM) is an alkylating chemical warfare agent that is responsible for a variety of cell and tissue damage, including inflammation, blister formation, and impaired wound healing (Kehe et al., 2009). Following exposure, SM causes injury to the eyes, respiratory system, and skin. In particular, skin wounds are of great concern because the healing process of these wounds is prolonged and requires in extreme cases up to months of hospitalization. Accordingly, treatment is cost expensive and might, in case of mass casualties, overburden the entire medical system. Beside symptomatic measures, therapeutic options primarily focus on ablation of the injured skin (Rice, 2003). Specific therapy options do not exist until today.

In general, wound healing can be divided into four sections (Bauer et al., 2005) and is started with the coagulation phase at the time of the injury, which is characterized by sealing the wound with a hemostatic plug and the first release of chemokines. During the second, inflammatory phase, lymphocytes and monocytes migrate by chemotaxis into the affected area and release further growth factors, which induce the third, proliferative phase. During this phase, fibroblasts and endothelial cells are activated and build collagen fibers or new vessels, respectively. The last fourth section is characterized by change into cicatricial tissue. During wound healing, vascularization (by vasculogenesis and angiogenesis) is regarded as most important for building granulation tissue (Bauer et al., 2005) and is mainly assigned to the third phase (proliferative phase), whereas during the inflammatory phase, angiogenic signals are set. If the formation of granulation tissue is affected, wound healing is mostly impaired (Li et al., 2003). Vascularization is determined by two different processes: new vessels can develop by sprouting out of already existing vessels (angiogenesis) (Risau, 1997; Schmidt et al., 2007) or blood vessel can form de novo from endothelial progenitor cells (EPC) (vasculogenesis) (Flamme et al., 1997; Schmidt et al., 2007). It has been demonstrated that EPC can be found in blood circulation of adult organisms and that these cells are involved in vascularization either by vasculogenesis or angiogenesis (Ribatti et al., 2001). In previous studies, it was shown that alkylating substances have negative influence on wound healing (Schlag and Kettelhack, 1989) and have an impact on angiogenesis even at nontoxic concentrations (Albertsson et al., 2003). Research concerning improvement of wound healing after SM exposure foremost deals with keratinocytes and fibroblasts, although it could be shown that...
endothelial cells can be seriously damaged by SM (Albertsson et al., 2003; Dabrowska et al., 1996; Schmidt et al., 2009) and that endothelial cells and their precursors are essential for wound healing (Bauer et al., 2006). A suitable in vitro model for investigating vascularization is the embryoid body (EB) model (Ribatti et al., 2001; Risau et al., 1988; Schmidt et al., 2005, 2006), which is derived from murine embryonic stem (ES) cells (Feraud et al., 2003). In EBs, vascularization processes are directly comparable to in vivo mouse models (Muller-Ehmsen et al., 2006). In previous studies, we demonstrated a negative effect of chlorambucil (a nitrogen mustard derivate) on proliferation, apoptosis, and endothelial tube formation in mouse EBs (Schmidt et al., 2009). But until today, nothing is known about the effect of SM on endothelial tube formation. Therefore, we analyzed the effect of 30 and 100μM SM on endothelial tube formation, apoptosis, and proliferation in EBs. As the initial formation of radicals (reactive oxygen species [ROS] and reactive nitrogen species) is discussed to be involved in the pathophysiology of SM toxicity (Gould et al., 2009; Naghii, 2002; Steinritz et al., 2009) and recent data have revealed the predominant role of NO and ROS in both angiogenesis and vasculogenesis (Duda et al., 2004; Schmelter et al., 2006), we evaluated the effect of ROS scavengers (z-linolenic acid [ALA] and N-acetyl cysteine [NAC]) on apoptosis, proliferation, and differentiation in the same experimental setup.

**MATERIALS AND METHODS**

**Chemicals.** All chemicals were reagent grade and purchased from Sigma, unless otherwise indicated.

**Cell culture/EB.** Mouse blastocyst-derived ES cells were established and maintained in culture as described previously (Doetschman et al., 1985). Briefly, ES cells were cultured on a fibroblast feeder layer in Dulbecco’s modified Eagle medium (DMEM) supplemented with 15% heat-inactivated fetal calf serum (FCS), 50 U/mL penicillin, 50 U/mL streptomycin, 200μg/mL L-glutamine, 100μg/mL β-mercaptoethanol, and 1% Eagle’s minimum medium (MEM) (GIBCO-BRL, Gaithersburg). ES cells were maintained in the undifferentiated stage in the presence of the cytokine leukemia-inhibiting factor (1000 U/mL; GIBCO-BRL, Gaithersburg). While hanging from the lid of the culture dish for 2 days (0–2 day), formation of EBs was allowed. The 100μM SM was regarded as vesicating dose for epithelial cells (Smith et al., 1993). Moreover, both concentrations are in the range of LD50 of immortalized endothelial cells (ISO-HAS 1) when cell viability is determined 24 or 48 h, respectively, after SM exposure (Kehe, unpublished data). Because of the complexity of the chosen EB model, a determination and validation of a complete dose-response curve was beyond the scope of this study. At predetermined points in time (Fig. 1), EBs were exposed to SM dissolved in 5 μL/mL et in MEM without FCS for 30 min. Solvent control group was treated with 5 μL/mL Et for 30 min. After exposure, EBs were washed with PBS, incubated in 0.5 ml DMEM medium plus 15% FCS, and were allowed to grow until the seventh day.

**Experimental design.** Changes of endothelial tube formation, proliferation, and apoptosis were always analyzed on day 7 of EBs’ growth (Fig. 1). Exposure of EBs with SM and treatment with NAC or ALA were conducted at designated points in time (days 0, 3, or 6) (Fig. 1) for 1 h (NAC) or for 24 h (ALA).

**NAC and ALA treatment.** To investigate the effect of ROS scavengers, EBs (n = 4 per point in time and treatment) were incubated with NAC (20mM, 1 h; Sigma-Aldrich) or ALA (15 ng/mL, 24 h; Sigma-Aldrich). After incubation with either NAC or ALA, medium was changed and cells were allowed to grow until day 7.

**Immunofluorescence stainings.** All EBs were fixed on day 7 after plating with 4% paraformaldehyde in 0.1M PBS for 25 min and washed three times with 0.1M PBS. Cells were permeabilized with 0.25% Triton-X 100 and 0.5M NH4Cl in Tris-buffered saline (TBS) for 10 min. For blocking, probes were incubated with 5% bovine serum albumin (BSA) in TBS (1 h, room temperature). The primary antibodies were diluted in 0.8% BSA and incubated overnight at 4°C. As primary antibodies, rat anti-mouse CD31 (PECAM-1, 1:800, rat; Pharmingen, San Diego), anti-Ki67 (proliferation marker, 1:250, rabbit; Abcam, Cambridge, UK), and anti-activated caspase-3 antibody (1:500; rabbit; BD Pharmingen) were used. We performed double stainings with PECAM-1 and KI67 or PECAM-1 and active caspase-3, respectively. EBs were washed four times with TBS and incubated with the fluorescent secondary antibody corresponding to the primary antibody host’s species. As secondary antibodies, goat anti-rat immunoglobulin G (IgG) biotinylated antibody (1:500, #BPN1005; GE Healthcare Life Sciences, Buckinghamshire, UK) followed by an incubation with streptavidin ALEXA Fluor 555-conjugated antibody (1:500, 485-21381; Life Technologies Corporation, Carlsbad, CA; exitation (ex.) 553 nm, emission (em.) 565 nm) and CY2-conjugated goat anti-rabbit IgG antibody (1:500, #111-225-047; Jackson ImmunoResearch, Suffolk, UK; ex. 494 nm, em. 510 nm) were used. The incubation with secondary antibodies was done in TBS for 1 h at room temperature in the dark. For negative control, experiments were done with secondary antibodies only.

**Analysis of endothelial tube formation.** To evaluate the effect of SM exposure on endothelial tube formation, EBs were exposed to SM as described above. To differentiate endothelial cells from other cell types, PECAM-1
(CD31) was used as an endothelial cell surface marker, which is already expressed in endothelial precursor cells as well as in embryonic vessels (Müller-Ehmsen et al., 2006; DeLisser et al., 1994; Vecchi et al., 1994). Intact endothelial tubes were manually counted using a fluorescence microscope (Axiohot, Zeiss, Germany; Cy2 filter: ex. 494/em. 510 nm; Cy3 filter: ex. 553/em. 565 nm). For evaluation, every vessel-like, PECAM-positive structure was counted of SM-treated as well as untreated EBs. Sprouts or tubes composed of at least three endothelial cells were considered as tube-like structures. Statistical analyses were performed as described below.

Analysis of apoptosis and proliferation. To evaluate the effect of SM on apoptosis and proliferation, immunofluorescence stainings against active caspase-3 (apoptosis) or Ki67 (proliferation) have been conducted. In sum, four EBs for each condition (SM or solvent control) and point in time (0, 3, or 6 days) have been double labeled against PECAM-1 (ALEXA Fluor 555) and either active caspase-3 (Cy2) or Ki67 (Cy2), respectively. Positive nuclei (for activated caspase-3 or Ki67, respectively) within 50 randomly chosen vessel-like tubes (PECAM-1 positive) have been counted using a fluorescence microscope (Axiohot, Zeiss, Germany; Cy2 filter: ex. 494/em. 510 nm; Cy3 filter: ex. 553/em. 565 nm) as described previously (Laude et al., 2004). If less than 50 PECAM-1-positive endothelial tubes could be identified, the counted positive cells have been extrapolated to 50 endothelial tubes.

Statistics. For statistical analysis, data out of four biological experiments for each condition and point in time were analyzed (n = 4). Analysis of endothelial tube formation can be recognized as a special case. Because of the immunohistochemistry double-labeling procedure, data out of eight EBs (n = 8) stained against PECAM-1 for each condition and point in time were available (nKi67 = 4 + nactivated caspase-3 = 4 → nPECAM-1 = 8). GraphPadPrism v5.0 software and two-way ANOVA were used to analyze the data. a-Values of 0.05 or below were regarded as significant. Details of the statistical evaluations are provided in Supplementary data 1.

RESULTS

Effect of SM on Endothelial Tube Formation, Proliferation, and Apoptosis in EBs

Under influence of SM, the number of endothelial tubes formed in EBs decreased dramatically compared with solvent control group (ethanol = Et) (Figs. 2A and 2B and 3A). This effect was significant at every point in time with a maximum reduction of endothelial tube formation when EBs were treated with 100 μM SM on day 0 (−96%). Although the effect of only two concentrations of SM on vessel formation in EBs was analyzed, both SM exposure groups showed a dose-related effect. As a result of previous studies (Schmidt et al., 2009), Et as solvent control did not affect endothelial tube formation compared with medium control (data not shown).

Ki67, which is expressed during cell division, was taken as marker of proliferation. In the solvent control group (Et), no changes of Ki67 could be detected in time course of EB development. Exposure of EBs to 30 and 100 μM SM tended to increase proliferation when administered on day 0 and day 3 of treatment (Fig. 3B). Activated caspase-3 was used as a common downstream marker for apoptosis. In the solvent control group, no changes of activated caspase-3 levels were detectable in time course. After SM exposure, significant changes of activated caspase-3 levels in EBs were detectable (Fig. 3C). Exposure to SM (30 and 100 μM) on day 0 tended to increase apoptosis in a dose-dependent manner, but changes of activated caspase-3 did not reach significance level. Exposures on day 3 to both 30 and 100 μM SM significantly increased apoptosis. In case of day 6 exposures, only 100 μM SM was able to significantly increase apoptosis, whereas 30 μM SM did not affect levels of activated caspase-3.
Effect of NAC and ALA on Endothelial Tube Formation, Proliferation, and Apoptosis in EBs

Both NAC and ALA affected endothelial tube formation in developing EBs compared with solvent control (Fig. 4A).

FIG. 3. Influence of 30 and 100μM SM on endothelial tube formation (3A), proliferation (3B), and apoptosis (3C). Et was used as solvent control. (A) SM significantly affects endothelial tube formation at any point in time and at both concentrations tested. When EBs are exposed to 100μM SM on day 0, a reduction of ~96% is detectable 7 days later. (B) SM tends to increase proliferation as measured by Ki67 immunohistochemistry, but changes did not reach level of significance. (C) When administered on day 3, 30 and 100μM SM significantly increased activated caspase-3. Exposure on day 6 led to significant caspase-3 activation only in the 100μM SM group.

FIG. 4. Influence of NAC (20mM) or ALA (15 ng/ml) on endothelial tube formation, proliferation (Ki67), and apoptosis (activated caspase-3) in EBs. Both, NAC and ALA, have influence on endothelial tube formation and proliferation. (A) Exposure of EBs on day 0 with NAC decreased endothelial tube formation, whereas ALA increased the amount of endothelial tubes detected on day 7. Exposure of EBs on day 3 with ALA decreased endothelial tube formation. When treatment was conducted on day 6, both ALA and NAC decreased endothelial tube formation. (B) Only ALA increased proliferation when treatment took place at day 3 of EB development. (C) Apoptosis was not affected by NAC or ALA.

Effect of NAC and ALA on Endothelial Tube Formation, Proliferation, and Apoptosis in EBs

Both NAC and ALA affected endothelial tube formation in developing EBs compared with solvent control (Fig. 4A).
Administered on day 0, ALA significantly increased endothelial tube formation, whereas NAC led to significant decrease. Moreover, ALA decreased endothelial tube formation when applied on day 3. Both NAC and ALA led to significant decrease of endothelial tube formation when administered on day 6. Ki67 was only affected by ALA when given on day 3. At this point in time, a significant increase of proliferation could be detected. Proliferation was not affected by ALA when applied on day 0 or day 6. NAC had no effect on proliferation (Fig. 4B). Active caspase-3 levels were not affected after administration on day 0 or day 6. At this point in time, proliferation was not affected by NAC or ALA (Fig. 5B).

**Effect of NAC and ALA on Endothelial Tube Formation, Proliferation, and Apoptosis in EBs after SM Exposure**

Treatment of SM-exposed EBs with NAC or ALA partially counteracted the effect of 100μM SM-induced reduction of endothelial tube formation when SM exposure and NAC treatment were carried out on day 6 of EB development (Fig. 5A). At this point in time, NAC led to a significant increase in endothelial tube formation. ALA also tended to increase the amount of endothelial tubes at this point in time, but this increase did not reach the level of significance. Neither NAC nor ALA showed significant effects in the 30μM SM group after administration on day 6. Given at earlier points in time, neither NAC nor ALA increased the number of endothelial tubes. Proliferation was only increased when EBs exposed with 30μM SM were treated with ALA on day 3. At all other points in time, proliferation was not affected by NAC or ALA (Fig. 5B).

Apoptosis as measured by active caspase-3 levels showed time-dependent changes after SM exposure and treatment with NAC or ALA, respectively (Fig. 5C). Given at day 0, both NAC and ALA decreased activated caspase-3 levels in 100μM SM-exposed EBs, but only NAC treatment reached level of significance. After treatment on day 3, no significant changes were detectable. At this point in time, ALA tended to decrease activated caspase-3 level in 100μM-exposed EBs comparable to day 0, whereas NAC tended to decrease the level of activated caspase-3 in 30μM-exposed EBs. Given on day 6, both substances similarly tended to decrease level of activated caspase-3 in 100μM-exposed EBs, but changes did not reach level of significance.

**DISCUSSION**

This study has shown for the first time that SM has a negative effect on endothelial tube formation in murine EBs, which is a suitable model for angiogenesis and vasculogenesis (Ribatti et al., 2001; Risau et al., 1988). Furthermore, we were able to demonstrate that ROS scavengers NAC and ALA showed a temporary but not a long-lasting protective effect regarding endothelial tube formation after SM exposure.

These findings are of particular interest as the vascular system has been identified as crucial to wound healing (Bauer et al., 2005). SM-induced chemical burns are characterized by impaired wound healing (Kehe et al., 2009; Rice, 2003). Additionally, after the long-lasting wound healing processes are completed, the result is often unsatisfactory as it was reported by patients who had contact with SM years ago (Hassankhani et al., 2010). Furthermore, endothelial malformations like cherry angiomas or pathological ocular vessels are observable in patients years after SM exposure (Emadi et al., 2008; Hefazi et al., 2006). In general, the formation of granulation tissue is required for a successful healing process of skin wounds. This specialized tissue is composed of fibroblasts and newly formed blood vessels, which can develop by sprouting out of existing vessels (angiogenesis) (Risau, 1997; Schmidt et al., 2007) or can be formed de novo from EPC (vasculogenesis) (Flamme et al., 1997; Schmidt et al., 2007). This vascularization process by vasculogenesis and angiogenesis is the key step in wound healing (Asahara et al., 1999) and is a complex process in which many key mechanisms are involved (proliferation, apoptosis, and migration) (Patan et al., 2001; Risau and Flamme, 1995). It was described that alkylating substances influence vascularization and as a consequence have impact on wound healing (Eldad et al., 1998; Rice, 2003). Recent studies have demonstrated that the EB model is suitable to explore vascularization processes like during wound healing processes (Ribatti et al., 2001; Risau et al., 1988; Schmidt et al., 2005, 2006). In a previous study, we reported the negative influence of the nitrogen mustard derivate chlorambucil on early vascular development in EBs (Schmidt et al., 2009). In this study, we focused on the effect of SM (30 and 100μM), concentrations that were found in the range of LD50 of immortalized human endothelial cells (ISO-HAS 1) determined by a vitality assay (XTT) 48 or 24 h postexposure, respectively (unpublished data), on early vascular development in murine EBs. Moreover, 100μM SM is described as the lower vesicating concentration (Smith et al., 1993). To account for the greater susceptibility of endothelial cells and their subepidermal position, we estimated 30μM SM as another relevant concentration. EPC derived from murine ES cells are mainly responsible for de novo building of endothelial tubes during the first days (days 0–3) of EB development (vasculogenesis). At later developmental stages (days 3–6), beside vasculogenesis, mainly angiogenesis is responsible for modeling vascular structures because of sprouting of endothelial cells out of formerly built vessels (Jakobsson et al., 2007). In our experiments, SM had strong impact on vascular development in EBs as seen by reduction of vascular structure formation. This effect was obvious after SM exposure at any point in time (days 0–6). Owing to the complexity of the EB model, the determination of a complete dose-response curve was out of the scope of our study, but the two used SM concentrations showed concentration-dependent characteristics with regard to reduction of vascular structures. Because of the fact that during EB development different cell types are present (stem cells, EPC, and differentiated...
FIG. 5. Effect of NAC or ALA on endothelial tube formation, proliferation, and apoptosis in SM-exposed EBs. NAC and ALA partially counteracted SM-induced reduction of vessel formation. (A) When EBs were exposed on day 0 or day 3, treatment with ALA or NAC had no effect on endothelial tube formation. Only when EBs were exposed to 100 μM SM on day 6, treatment with NAC significantly restored endothelial tube formation. ALA also tended to restore endothelial tube formation at this point in time but did not reach level of significance. (B) Proliferation of SM-exposed EBs was only affected in EBs exposed to 30 μM SM on day 3 and treatment with ALA. (C) At day 0, treatment of SM-exposed EBs significantly decreased apoptosis as measured by activated caspase-3 levels. ALA tended into the same direction but did not reach level of significance. At day 3, no significant effects were detectable. NAC tended to decrease apoptosis in 30 μM SM–exposed EBs, whereas ALA tended to decrease apoptosis in 100 μM-exposed EBs. When EBs were exposed to 100 μM SM on day 6, treatment with NAC and ALA both tended to decrease apoptosis, but values did not reach level of significance.
endothelial cells) (Conley et al., 2005), our results point to direct influence of SM to all these cell types. Furthermore, in our experimental design, EBs exposed to SM on day 0 were allowed to grow until day 7 so that cells had the opportunity to recover from the initial damage. On the other hand, severely damaged cells had been eliminated at early points in time, e.g., by apoptosis, as observed in our experiments. We have been able to demonstrate that SM-induced damage on day 0 (amount of vessels and apoptosis) is still detectable on day 7. At this point in time, it is unlikely that severely damaged cells from day 0 can still be found in the EB. These findings point to long-lasting damaging processes, and it can be speculated that next generation cells still show SM-induced damages.

In our experiments, the reduction of vascular structures after SM exposure is accompanied by a slight, but not significant, increase of the proliferation marker Ki67. The increase of proliferation seems to be in contrast to the negatively affected vascular development. However, the increase of proliferation is weak and fails to reach significance. The slight increase may reflect the proliferative activity of marginally affected cells, aimed to compensate the apoptotic elimination of those cells that had been severely damaged. Regarding apoptosis, it can be noted that because of SM exposure, there is a significant increase of activated caspase-3 when exposure occurs on day 3 and day 6 in both SM groups and a slight, but not significant, increase following day 0 exposures. Caspase-3 belongs to the caspase family and has been implicated to play an important role in apoptosis as the enzyme is recognized to be the main effector caspase during apoptotic cell death. Caspase-3 is processed in cells undergoing apoptosis by self-proteolysis and/or cleavage by other upstream proteases (e.g., caspases-8, -9, and -10). Once activated, active caspase-3 proteolytically cleaves relevant targets in the cells (e.g., Poly [ADP-ribose] polymerase) and finally leads to cell death.

This in turn can explain the reduction of vascular structures. Again we have hints that not only stem cells and progenitor cells but also differentiated endothelial cells are long-lasting affected because EBs exposed to SM on day 0 still tended to show changes of proliferation and apoptosis on day 7. Moreover, exposure of murine EBs to 30μM SM led to a detectable increase of apoptosis only on day 3 of EB exposure, whereas exposure to 100μM SM already significantly increased the level of activated caspase-3 when given on day 6, i.e., only 1 day before analysis. This may suggest that a higher concentration of SM (100μM) initiates apoptosis already 1 day after treatment and EB do not recover from this initial activation of apoptotic pathways, whereas a lower SM concentration (30μM) does not induce apoptotic pathways that are detectable 1 day after exposure but only 3 days after exposure.

It is discussed that oxidative and nitrosative stress is involved in the pathophysiology of SM toxicity (Naghii, 2002; Gould et al., 2009; Paromov et al., 2007; Steinritz et al., 2009). We therefore tested the radical scavengers NAC and ALA. Again owing to the complexity of the EB model, we used established single concentrations and treatment periods of the two scavengers. In two earlier studies, we had determined 10mM NAC as an appropriate dose to protect immortalized keratinocytes from SM toxicity (Balszuweit, Oks, Steinritz, Thiermann, and Kehe, submitted; Oks, Balszuweit, Thiermann, and Kehe, submitted). As the EB is a three-dimensional model and contains multiple layers of cells (as opposed to the monolayer of cells, used in previous studies), we decided to increase the NAC dose to 20mM. The dose for ALA (15 ng/ml which is 53nM) was chosen after preliminary experiments had shown that ALA, when used in micromolar concentrations (as it is common in monolayer cell culture experiments), had multiple effects on the EB model. Even in the low concentration of 15 ng/ml, used in our study, ALA itself without any SM exposure had partially significant effects on endothelial tube formation and proliferation (Figs. 4A and 4B).

To limit those effects, we chose not to increase the dosage any further. Even though the concentration in our experiments is low, it still produced protective effects against SM toxicity, which came close to the level of significance. Both scavengers themselves affected vessel formation in EBs. This result is not unexpected as it is known that ROS modulate endothelial and vascular functions at multiple levels (Kunsch and Medford, 1999; Wolin, 2009), and physiological levels of ROS are considered as important for vascular development (Irani, 2000).

Treatment of 100μM SM–exposed EBs with NAC or ALA, respectively, restored formation of vessels when treatment of EBs and treatment with antioxidants were performed on day 6. At all other points in time, no effect was observable. These findings suggest that only the higher used SM concentration may lead to a distinct ROS formation and that mainly differentiated cells can be protected by NAC and ALA because these cells are predominant at EBs at the developmental stage on day 6.

It is important to emphasize that the protective effect of NAC and ALA seems not to be long lasting because SM-exposed EBs on day 0 and day 3 are not protected as the reduction of vessels cannot be counteracted. This means in turn that short administration of antioxidants will only lead to a short time period of protection. It can be speculated that only continuous treatment with NAC or ALA might be beneficial for wound healing processes after SM exposure. This hypothesis has to be proved in further studies.

Taken together, our results showed that SM has negative influence of vascularization in the EB model. ROS scavengers (NAC or ALA) partially counteracted this effect. Both substances showed temporary, but not long-lasting, effects. It could be possible that these substances have beneficial effects for wound healing processes after SM exposure when continuously administered.

**SUPPLEMENTARY DATA**

Supplementary data are available online at http://toxsci.oxfordjournals.org/.

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**Reference:**

Conley et al., 2005; Gould et al., 2009; Paromov et al., 2007; Steinritz et al., 2009.
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