c-Abl Is Required for the Development of Hyperoxia-induced Retinopathy

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Abstract

The requirement for the nonreceptor tyrosine kinase c-abl in the pathogenesis of retinopathy of prematurity (ROP) was examined using the mouse model for ROP and c-abl–deficient mice. Hyperoxia-induced retinal neovascularization was observed in wild-type and heterozygous mice but animals that were homozygous null for c-abl did not develop a vasoproliferative retinopathy in response to hyperoxia. Two gene products, endothelin-1 (ET-1) and vascular endothelial growth factor (VEGF), have been implicated in the pathogenesis of ROP. The mRNA expression of ET-1 and VEGF was assessed in mice maintained in normoxia and in hyperoxia-exposed mice. ET-1 mRNA levels were unchanged in wild-type mice throughout the hyperoxia treatment, suggesting that ET-1 mRNA expression is not regulated by the increase in inspired oxygen. In wild-type mice maintained in room air, VEGF mRNA levels rose threefold from postnatal day 6 (P6) to P17. When wild-type mice were treated with the hyperoxia regimen, a fivefold decrease in VEGF mRNA expression was observed from P7 to P16. However, retinal VEGF expression in hyperoxia-treated homozygous null mice did not decrease and remained at control levels. These data suggest that c-abl is required for the hyperoxia-induced retinal neovascularization and hyperoxia-induced decrease in VEGF mRNA levels.

Key words: retinal angiogenesis • oxidative stress • DNA damage • nonreceptor tyrosine kinase • vascular endothelial growth factor

Introduction

Retinopathy of prematurity (ROP) is a vasoproliferative retinopathy of premature infants that results in severe visual impairment and blindness (1, 2). Animal models that replicate clinical manifestations of ROP have been used to characterize the roles of vascular endothelial growth factor (VEGF [3–6]), VEGF receptors (flk-1 and flt-1 [7]), and hypoxia inducible factor-1 (HIF-1 [8]) in the pathogenesis of ROP. Exposure to hyperoxia results in the downregulation of VEGF production, vasoconstriction, cessation of retinal blood vessel growth, and loss of capillary beds (9). The vasoconstrictive response to hyperoxia has been proposed to be endothelin-1 (ET-1) dependent (10–13). The expression of ET mRNA in response to hyperoxia is unknown. The drop in retinal vessel density occurs via apoptosis and has been attributed to decreased levels of VEGF mRNA, as VEGF is required for the viability of vascular endothelial cells (4, 5). The resulting nonperfused retinal tissue responds by increasing its VEGF production and new blood vessel growth (14, 15). VEGF-induced angiogenesis is Src dependent and Src kinase activity protects newly formed vessels from apoptosis (16).

The mechanism by which oxygen levels elicit changes in VEGF expression is poorly understood. However, reactive oxygen intermediates have been implicated in the pathogenesis of ROP (17). The administration of antioxidants to mice exposed to a hyperoxia regimen prevented the development of retinopathy (18). In addition, there is evidence that HIF-1 binds to DNA damaged by oxidation and may affect the repair rate of oxidation-induced strand breaks (19).
c-Abl is a protooncogenic nonreceptor tyrosine kinase with an NH$_2$-terminal domain that is homologous to Src family members and a unique COOH-terminal region (20). Mutant forms of c-abl have been characterized in leukemias of mice and humans (21). There are two isoforms of c-abl that differ at the NH$_2$-terminal sequences due to alternate 5’ exons (20, 22, 23). The c-abl protein is ubiquitously expressed and localizes to the cytoplasm and nucleus where it has distinct roles (20, 23). Effects of c-abl on the cell cycle, apoptosis, and cellular transformation depend on protein interactions with its various functional domains: Src homology (SH)3, SH2, tyrosine kinase domain, proline-rich binding sites, and actin binding domain. c-Abl participates in the signal transduction pathways induced by growth factors (24), integrins (25), and genotoxic stresses such as ionizing radiation and hydrogen peroxide (26–29). c-Abl tyrosine kinase activity may be enhanced by Src kinases that directly phosphorylate the kinase domain of c-abl (24). Both VEGF and ET-1 receptor signaling require c-Src–dependent pathways (16, 30), and these cytokines have been implicated in the pathogenesis of oxygen-induced neovascularization. Because c-abl participates in the cellular response to oxidation-induced DNA damage and in Src kinase–dependent signaling pathways, we hypothesized that c-abl may be involved in the angiogenic response to hyperoxia.

The in vivo requirement of c-abl for hyperoxia-induced neovascularization was investigated using an established mouse model for ROP and mice carrying knockout mutations of the c-abl gene (9, 31). We report that, unlike wild-type (wt) control animals, mice homozygous for a c-abl null mutation do not develop neovascular retinopathy in response to hyperoxia. To determine if retinal VEGF and ET-1 expression in response to hyperoxia are aberrantly regulated, their mRNA levels were analyzed using RNase protection assays. We observe that ET-1 mRNA levels in retinas of wt and homozygous c-abl null animals do not differ, and exposure to the hyperoxia regimen does not alter ET-1 mRNA levels in either wt or c-abl null animals. As reported by others, we find that retinal VEGF mRNA levels in wt animals change in response to hyperoxia. However, VEGF mRNA levels remained unchanged in oxygen-exposed c-abl null mice compared with control animals.
ET-1 and VEGF riboprobes are 584 and 615 nt, respectively. Transcription reactions proceeded according to instructions provided in the MAXIscript transcription kits (Ambion). For RNase Protection assays, ET-1, VEGF, and actin riboprobes (200 pg) were coprecipitated with total RNA (3–5 µg) at a 100-fold molar excess of probe to target mRNAs and hybridized in 80% formamide hybridization buffer (RPAIII; Ambion) overnight at 42°C. Total RNA was isolated from dissected retinas using RNAzol B (Tel-test). Unhybridized probes were digested with an RNase A/RNase T1 mix (RPAIII; Ambion) and protected probes were precipitated with yeast carrier RNA (10 µg). Digestion of the polylinker sequence present in protected riboprobes generates shortened fragments: actin, 250 bp; ET-1, 484 bp; VEGF, 410 bp. Precipitated RNA fragments were resolved on a 7 M urea-5% polyacrylamide gel. Bands were visualized and quantitated by PhosphorImager analysis (Molecular Dynamics).

Results

c-Abl–deficient Mice Do Not Develop Retinal Neovascularization in Response to Hyperoxia. To determine if mice deficient in c-abl are susceptible to hyperoxia-induced neovascularization, a mouse model for ROP was employed (9). Matings of mice that were heterozygous for the c-abl null allele were established to generate control and experimental age-matched litters (31). Control animals were maintained in room air (normoxia) for the duration of the experiment, and the experimental group was exposed to a hyperoxia regimen (5, 9, 14). In this regimen, pups and their nursing mother were exposed to hyperoxia (75% oxygen) starting on P7 for 5 d followed by their return to normoxia on P12 for an additional 4–5 d. Retinas were harvested for examination of its vasculature by fluorescein angiography on

Figure 1. Flat-mounted retinas perfused with fluorescein-dextran from wt and c-abl homozygous null mice. Age-matched wt and c-abl−/− mice were either maintained in normoxia or exposed to 75% oxygen from P7 to P12 and then placed in room air from P12 to P20. The retinal vasculature of all animals was examined by fluorescein angiography on P17–20.
P17–20. Animals that were wt (Fig. 1) or heterozygous (data not shown) for c-abl developed a strong vasoproliferative response to the hyperoxia treatment when compared with wt or c-abl heterozygous animals kept in normoxia. However, retinas of c-abl homozygous null mice exposed to hyperoxia exhibit minimal to no retinopathy when compared with retinas prepared from all mice maintained in normoxia (Fig. 1). This suggested that c-abl is required for hyperoxia–induced retinopathy to develop.

A retinopathy scoring system was used to quantify the extent of vasoproliferation present in each retinal whole-mount (33). Two independent observers that were blinded to specimen identity examined each section. They assigned a numerical score from 0 to 4, where 4 corresponds to the maximal response, for each of the following categories: blood vessel growth, blood vessel tufts, extra retinal neovascularization, central vasoconstriction, retinal hemorrhage, and tortuosity of vessels. The scores for all six categories were totaled. When the code was broken, the total scores were tabulated and used to quantify the severity of the neovascular retinopathy developed by wt, +/−, and −/− mice. Retinas of wt or +/− mice exposed to hyperoxia exhibited predominantly vessel tufts, central vasoconstriction, and vessel tortuosity. Retinal hemorrhages and extra retinal vessels were minor aspects of the retinopathy. A scatter distribution of the total retinopathy scores obtained from wt, heterozygous, and homozygous c-abl null mice exposed to normoxia or hyperoxia was generated (Fig. 2). The median total scores for all the animals maintained in room air were zero. Upon exposure to hyperoxia, the wt showed an increase in the median score to 3.5, whereas the heterozygotes showed an increase to 2. The neovascular retinopathy developed in response to hyperoxia demonstrated a wide spread of total retinopathy scores. Nonparametric analysis of variance (Mann–Whitney test) revealed no significant difference between wt and heterozygote mice and a significant difference (P < 0.0001) between the normoxia and hyperoxia-exposed groups. In contrast, the median scores observed for homozygous null mice maintained in room air and exposed to hyperoxia were both zero. The median values observed for the homozygous animals showed a highly significant difference (P < 0.0001) from those of hyperoxia-exposed control (+/+ and +/−) mice. These data demonstrate that, taken together, control animals (+/+ and +/−) developed a moderate retinal neovascular response to hyperoxia as described previously, whereas homozygous c-abl null animals did not develop a retinopathy (9).

To confirm the data obtained using the subjective retinal scoring system, the nuclei of new blood vessels that extend beyond the inner limiting membrane into the vitreous were counted (9). The nuclei were observed in PAS-stained retinal frozen sections (Fig. 3 A). Averaged numbers of nuclei from retinas of mice exposed to normoxia and hyperoxia were determined (Fig. 3 B). The mean numbers of nuclei observed for wt and heterozygotes exposed to hyperoxia were 20 and 17, respectively, whereas negligible numbers (≤5) of neovascular nuclei were observed in retinas of mice (wt, +/−, and −/−) maintained in room air. The difference in the average numbers of nuclei between normoxia and hyperoxia-treated groups was significant (P < 0.001). In contrast, the mean number of nuclei counted in retinas of homozygous c-abl null mice exposed to hyperoxia was 5.5 as observed in normoxia control retinas. There is a significant difference (P < 0.002) in the means between homozygous (−/−) and control (+/+ and +/−) mice exposed to hyperoxia. The nuclei count data confirm the total retinopathy scoring values, demonstrating that the c-abl–deficient mice failed to develop hyperoxia–induced vasoproliferative retinopathy.

ET-1 mRNA Expression in wt Mice Is Constant throughout the Hyperoxia Regimen. Constriction of retinal vessels is characteristic of the retinopathy and has been proposed to be ET-1 mediated (13). However, there are no reports that

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Figure 2. Scatter distribution of total retinopathy scores for normoxia and hyperoxia–exposed wt and c-abl–deficient mice. The vasculature of flat-mounted P17 retinas from mice exposed to (A) room air or the hyperoxia regimen (B) was assessed using the total retinopathy scoring system. The number of retinas observed for each total retinopathy score is plotted. A total score of 0 indicates normal retinal vasculature and a total score of 17 reflects severe retinopathy. Arrows point to the median total score for each group of mice.
describe retinal ET-1 mRNA expression in response to oxygen or in the murine ROP model we employed. To determine if ET-1 mRNA expression is differentially regulated in wt mice by oxygen, we assessed ET-1 mRNA levels in retinas of normoxia control and hyperoxia-treated experimental animals using RNase protection assays. Retinas from P6 and P17 control (normoxia) mice and from P7 to P16 experimental mice, i.e., mice exposed to the hyperoxia regimen, were used (Fig. 4). ET-1 was found to be expressed constitutively and levels were unchanged in the normoxia and hyperoxia-exposed retinas harvested throughout the hyperoxia regimen. These data suggest that retinal ET-1 mRNA levels are refractory to changes in inspired oxygen tension.

Mice Deficient in c-abl Develop a Normal VEGF-induced Angiogenic Response. Because VEGF is involved in the development of hyperoxia-induced retinal vasoproliferation (5) and mice deficient in c-abl failed to develop hyperoxia-induced retinopathy, we sought to determine if mice that lack c-abl could respond normally to VEGF. To examine if homozygous c-abl null mice generate an angiogenic response to exogenously supplied VEGF, a corneal angiogenesis assay was employed (35). A PBS sham or VEGF-saturated Hydron pellet was implanted surgically in corneas of 3-wk-old wt and homozygous null mice. New blood vessels emerged from the limbal vasculature of wt and c-abl-deficient mice toward the VEGF-saturated pellet, whereas no vessels were observed in animals with sham pellets (Fig. 5). The density of angiogenic vessels emerging from the limbus toward the pellet was similar between +/+ and +/- mice. These observations demonstrate that c-abl activity is not required for VEGF-induced signal transduction and that the c-abl-/- mice responded normally to the ectopic administration of VEGF.

Figure 3. (A) Representative PAS-stained sections. Retinal sections from +/+, +/-, and +/- c-abl mice (P17) were compared between normoxia control and experimental animals exposed to the hyperoxia regimen. Arrows point to new blood vessels grown beyond the inner limiting membrane. (B) Numbers of extra retinal neovascular nuclei in PAS-stained retinal sections. P17–P20 retinas from wt and c-abl-deficient mice exposed to room air or the hyperoxia were sectioned and stained with PAS as shown in panel A. The total number of vascular nuclei extending outside the inner limiting membrane in 6-μm retinal cross-sections was counted. The average number of nuclei for number (n) of examined eyes and standard error bars are graphed.
VEGF mRNA Expression in wt Mice Is Regulated Differentially in Response to Hyperoxia. To better understand the effect of the hyperoxia treatment on VEGF mRNA expression, we first characterized VEGF mRNA levels in two wt mouse strains using RNase Protection assays. The C57Bl/6 strain used traditionally in the mouse ROP model and the hybrid 129Sv/Ev/H11003 C57Bl/6 strain in which the c-abl null allele is propagated were examined. Total RNA (5 μg) was hybridized with VEGF, ET-1, and actin riboprobes (arrows). Riboprobes alone were treated with (+) or without (−) RNase to verify that digestions were complete. The protected fragments were resolved by urea/SDS-PAGE and visualized by PhosphorImager. Band density was quantified using ImageQuant software. The normoxia and hyperoxia hybridization experiments were performed using riboprobes prepared on separate occasions. Therefore, the specific activities differed and expression levels between these two groups should not be directly compared.

Exposure to Hyperoxia Does Not Elicit Decreased VEGF mRNA Expression in Retinas of c-abl−deficient Mice. Because the homozygous null mice were protected from hyperoxia-induced retinal stress (Figs. 1–3), we sought to determine if differences in retinal VEGF mRNA expression among wt, heterozygous, and homozygous c-abl mice could be involved. Mice used for these experiments were of the hybrid strain. VEGF mRNA levels were assessed in P17 retinas of mice maintained in room air and of mice exposed to the hyperoxia regimen using RNase protection assays. VEGF mRNA expression in wt mice exposed to hyperoxia (Fig. 7, lanes 4 and 7) decreased sevenfold compared with the normoxia control level (Fig. 7, lane 3). In contrast, the VEGF mRNA levels in retinas of homozygous null animals exposed to the hyperoxia treatment remained consistently unchanged (Fig. 7, lanes 8 and 11) from levels observed in normoxia control retinas (Fig. 7, lanes 3 and 5). These data suggest that homozygous animals lacking any c-abl do not regulate VEGF mRNA expression in response to oxygen.

The VEGF expression levels in retinas of heterozygotes exposed to the hyperoxia regimen was variable as seen in repeat experiments (lanes 4 and 7) decreased sevenfold compared with the normoxia control level (Fig. 7, lane 3). In contrast, the VEGF mRNA levels in retinas of homozygous null animals exposed to the hyperoxia treatment remained consistently unchanged (Fig. 7, lanes 8 and 11) from levels observed in normoxia control retinas (Fig. 7, lanes 3 and 5). These data suggest that homozygous animals lacking any c-abl do not regulate VEGF mRNA expression in response to oxygen.
be a gene dosage effect for the reduction of VEGF mRNA levels mediated by one or two copies of c-abl.

**Discussion**

Our data demonstrate that c-abl is required for the development of vasoproliferative retinopathy induced by hyperoxia in the murine model of ROP. Oxygen-induced blood vessel growth, vessel tufts, central vasoconstriction, and vessel tortuosity were readily detected in retinal flatmounts of wt and c-abl heterozygous mice. Retinal hemorrhages and extra retinal neovascularization were not prominent features of the retinopathies. Mice that were homozygous null for c-abl did not develop the oxygen-induced retinal vascular obliteration or neovascularization that was observed in control mice. The specific role for c-abl in the retinal response to hyperoxia remains unknown. We speculate that c-abl contributes to the signaling network responsible for sensing and responding to intracellular oxidative effects.

Vasoconstriction of the central retinal vasculature has been described in animals treated with hyperoxia (9, 12) and ET-1 activity has been implicated in retinal vasoconstriction (10, 11, 13). Because it was unknown if hyperoxia induces altered ET-1 expression, we assessed retinal ET-1 mRNA levels in wt mice maintained in room air and exposed to the hyperoxia regimen. ET-1 mRNA levels remained constant in P6 and P17 retinas maintained in normoxia and did not change throughout the hyperoxia regimen. These data indicate that retinal ET-1 expression levels may not be regulated postnatally during retinal angiogenesis or change in response to increased levels of inspired oxygen. Nevertheless, a currently undefined role for constitutively expressed ET-1 may be important for normal retinal angiogenesis and vascular homeostasis.

To better understand why homozygous c-abl null mice were refractory to hyperoxia-induced stress as observed by their failure to develop a neovascular response to hyperoxia, we examined if VEGF-dependent signaling was aberrant in c-abl–deficient mice. A role for VEGF in oxygen-induced retinopathy has been documented by others (5, 7, 14, 15, 40, 41). Control and c-abl–deficient animals generated a robust neovascular response to VEGF-saturated corneal pellets indicating that c-abl is not necessary for VEGF-dependent signal transduction.

As the in vivo response to ectopically administered VEGF was intact in homozygous null mice, we analyzed VEGF mRNA expression. VEGF mRNA levels have been documented to be differentially regulated in the mouse model for ROP (5, 14, 41). Others and we observed a fall in VEGF mRNA levels when C57Bl/6 mice were exposed to 75% oxygen, whereas a rise in levels occurred when animals were returned to normoxia. It has been proposed that the relative hypoxia experienced when animals are removed from 75% oxygen and placed in normoxia induces an accumulation of VEGF mRNA (42, 43). Also, we characterized the VEGF mRNA levels in our wt hybrid (129Sv/Ev × C57Bl/6) mice throughout the hyperoxia regimen. The kinetics of VEGF mRNA expression induced by hyperoxia differed between wt C57Bl/6 and wt hybrid mice. The difference in gene regulation in response to oxygen among strains suggests that there may be multiple roles for VEGF in the development of oxygen-induced retinopathy. Recent results from the supplemental therapeutic oxygen for prethreshold (STOP)-ROP clinical trial in which supplemental oxygen therapy is administered to inhibit retinal neovascularization also raise the possibility that the role of VEGF in vasoproliferation is complex.

Because hyperoxia-induced retinopathy was not observed in c-abl–deficient mice and regulation of VEGF expression is important to the development of ROP, we determined whether c-abl knockout mice showed abnormal VEGF mRNA regulation when exposed to the hyperoxia regimen. Whereas hyperoxia-treated hybrid wt mice showed a large decrease in VEGF mRNA levels by P16, retinas of P16 hyperoxia-treated c-abl–deficient mice.
showed no such decrease. These data suggest that c-abl is required for the hyperoxia-induced downregulation of VEGF mRNA observed in the hybrid strain. Moreover, this observation implies that c-abl contributes to signal transduction pathways that regulate VEGF gene expression.

Taken together, our studies suggest that inhibition of c-abl activity may protect neonates from developing ROP. The therapeutic use of the c-abl tyrosine kinase inhibitor STI571 in the treatment of patients with chronic myelogenous leukemia has been shown to be promising (44). Experiments are currently underway to determine if administration of STI571 to wt mice can mimic the c-abl null phenotype and, thus, prevent the development of hyperoxia-induced retinopathy.

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