Mitochondrial Aldehyde Dehydrogenase 2 Regulates Revascularization in Chronic Ischemia
Potential Impact on the Development of Coronary Collateral Circulation

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Objective—Revascularization is an essential process to compensate for cardiac underperfusion and, therefore, preserves cardiac function in the face of chronic ischemic injury. Recent evidence suggested a vital role of aldehyde dehydrogenase 2 (ALDH2) in cardiac protection after ischemia. This study was designed to determine whether ALDH2 regulates chronic ischemia–induced angiogenesis and to explore the underlying mechanism involved. Moreover, the clinical impact of the ALDH2 mutant allele on the development of coronary collateral circulation (CCC) was evaluated.

Approach and Results—Mice limb ischemia was performed. Compared with wild-type, ALDH2 deletion significantly reduced perfusion recovery, small artery and capillary density, and increased muscle atrophy in this ischemic model. In vitro, ALDH2-knockdown reduced proliferation, migration and hypoxia triggered endothelial tube formation of endothelial cells, the effects of which were restored by ALDH2 transfection. Further examination revealed that ALDH2 regulated angiogenesis possibly through hypoxia-inducible factor-1α/vascular endothelial growth factor pathways. To further discern the role of ALDH2 deficiency in the function of bone marrow stem/progenitor cells, cross bone marrow transplantation was performed between wild-type and ALDH2-knockout mice. However, there was no significant improvement for wild-type bone marrow transplantation into knockout mice. ALDH2 genotyping was screened in 139 patients with chronic total occlusion recruited to Zhongshan Hospital (2011.10–2014.4). Patients with poor CCC (Rentrop 0–1; n=51) exhibited a higher frequency of the AA genotype than those with enriched CCC (Rentrop 2–3; n=88; 11.76% versus 1.14%; P=0.01). However, the AA group displayed less enriched CCC frequency in Logistic regression model when compared with the GG group (odds ratio=0.08; 95% confidence interval, 0.009–0.701; P=0.026). Furthermore, serum vascular endothelial growth factor level tended to be lower in patients with ALDH2 mutation.

Conclusions—This study demonstrated that ALDH2 possesses an intrinsic capacity to regulate angiogenesis via hypoxia-inducible factor-1α and vascular endothelial growth factor. Patients with ALDH2-deficient genotype displayed a higher risk of developing poor CCC. Therapeutic individualization based on ALDH2 allele distribution may thus improve the therapeutic benefit, especially in the East Asian decedents. (Arterioscler Thromb Vasc Biol. 2015;35:2196-2206. DOI: 10.1161/ATVBAHA.115.306012.)

Key Words: aldehyde dehydrogenase ▪ coronary occlusion ▪ endothelial progenitor cells ▪ hypoxia-inducible factor ▪ neovascularization

The reestablishment of blood vessels through the process of angiogenesis, or formation of new blood vessels, is critical to protect the ischemic injury and promote the recovery because of the essential role to deliver oxygen and nutrients. Specifically in cardiovascular system, a functional collateral circulation may counter ischemia to preserve ventricular function and to improve the overall prognosis. Despite the high prevalence of coronary collateral circulation (CCC) in coronary angiogram images from patients with coronary heart disease, particularly chronic total occlusion (CTO), the efficacy of angiogenesis varies from one patient to the next, creating a dramatic inconsistency in the clinical outcome. The underlying mechanisms responsible for such variability seems to be complex and still remains unknown.

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Mitochondria aldehyde dehydrogenase (ALDH) or ALDH2 is a key enzyme in eliminating toxic aldehydes by catalyzing their oxidation to nonreactive acids.13–15 Accumulating data from our laboratory and others indicated a key protective role for ALDH2 in ischemic injury.16–18 These data suggest that ALDH2 is involved in regulation of the microenvironment homeostasis under ischemia or hypoxia condition. Notably, a loss-of-function allele of ALDH2 is found in nearly half of East Asian or 8% of world populations, which may be correlated with various pathological processes, such as ischemia/reperfusion injury and cardiac remodeling.19–21 Nonetheless, it remains somewhat elusive whether ALDH2 plays a role in angiogenesis in chronic ischemia.

To this end, this study was designed to investigate the role of ALDH2 in angiogenesis and the underlying molecular basis behind, if any, chronic ischemia. It further prompted us to scrutinize the impact of the Glu504Lys mutation in the development of CCC in the clinical setting. Our results showed that ALDH2 deficiency impairs the angiogenesis process possibly by inhibiting the endothelial cell function and the hypoxia-inducible factor-1α (HIF-1α)/vascular endothelial growth factor (VEGF) signaling cascade. On the other side of the coin, overexpression of ALDH2 restores the angiogenesis process. The clinical data implicated that loss-of-function for ALDH2 because of genetic mutation may present as an unfavorable contributing factor for revascularization in patients with CTO. Therefore, targeting on ALDH2 activity might serve as a potential therapeutic strategy for individualized ischemic injury.

**Materials and Methods**

Materials and Methods are available in the online-only Data Supplement.

**Results**

**ALDH2 Deficiency Deteriorates Perfusion Recovery and Revascularization in Chronic Hindlimb Ischemia, Leading to Promoted Gastrocnemius Muscle Atrophy**

To examine the effect of ALDH2 on perfusion recovery ability in chronic ischemic injury, a mouse ischemic limb model was used in ALDH2-knockout (KO) and wild-type (WT) mice (Figure 1A). The perfusion was assayed using the laser Doppler perfusion imaging on days 1, 7, and 21 after the femoral artery ligation surgery (Figure 1B). Our results indicated that ALDH2-KO mice displayed reduced perfusion signals (red and orange) associated with more pronounced nonperfusion signals (blue and green) in ischemic limbs compared with those from WT mice on days 7 and 21 (but not on day 1). Quantitatively, we calculated the ratio of perfusion between ischemic and nonischemic limbs for each mouse (Figure 1C). Our data revealed that the perfusion ratio achieved 82.2% at the 3-week point in WT group. In comparison, only 59.3% perfusion ratio was found in ALDH2-KO group, whereas there were no significant difference noted between the 2 groups on day 1, indicating a slower recovery of limb circulation (P<0.05; Figure 1B and 1C). These observations demonstrate that the perfusion recovery of ischemic hindlimb may be impaired by deficient ALDH2 expression.

To determine if deteriorated tissue perfusion was mediated, in part, through reduced revascularization, small artery density was determined 21 days after hindlimb surgery (Figure 1A and 1D). Using immunofluorescence staining, small artery was detected by FITC labeled anti-α smooth muscle actin antibody in injured limbs. Less small artery density was found in the injured limbs of ALDH2-KO mice compared with that from the WT mice (22.8±5.2 versus 12.1±5.6 number/field in WT and ALDH2-KO mice, respectively; P<0.05; Figure 1D and 1E). To assess the angiogenesis effect, capillary density was determined at the same time (Figure 1A and 1F). Using immunofluorescence stain, capillary density was detected by the anti-CD31 antibody in hindlimbs. The micrographs showed that WT ischemic limb displayed reduced capillary density (less red CD31 (+) signal) compared with WT nonischemic limb. ALDH2-KO ischemic limb displayed the lowest CD31 (+) cell density among the 4 groups. The CD31 (+) cells were significantly less in KO-ischemic group when compared with WT-ischemic group (P<0.05), although the nonischemic limb of ALDH2-KO mice showed equivalent amount of CD31 (+) cells compared with WT mice (0.757±0.036 versus 0.580±0.008 CD31 (+)/DAPI in (Figure 1E; P<0.05) WT nonischemic and ischemic limb, and 0.766±0.028 versus 0.402±0.040 (P<0.05) in KO nonischemic and ischemic limb.

Chronic ischemic insult is usually linked to permanent damage to the limb, manifested as muscle atrophy and loss of limb function.22 Therefore, gastrocnemius muscle atrophy rate was assessed in ischemic limbs, shown as the ratio of weight of ischemic and nonischemic gastrocnemius muscles. Our data revealed somewhat comparable muscle atrophy in ischemic and nonischemic gastrocnemius muscles. Despite the perfusion recovered to >50% of that of the noninjured limb at the 3-week time point. ALDH2-KO mice presented a 25.2% loss of gastrocnemius muscle weight when compared with a 17.5% loss in muscle weight in WT mice (P<0.05; Figure 1F).

Taken together, these data demonstrate that deficient ALDH2 expression directly impedes the revascularization and perfusion, leading to worsened tissue repair and functional restoration in chronic ischemia.

**ALDH2 Deficiency Impairs Tube-Like Construction Formation, Migration, and Proliferation In Vitro**

To further determine the regulatory role of ALDH2 in angiogenesis in chronic ischemic microenvironment, the
endothelial cell function was evaluated. First, the tube-like construction formation assay was performed in rat cardiac microvascular endothelial cells (Figure 2A). To mimic WT and ALDH2-knockdown endothelium, scramble and ALDH2-siRNA were transfected, respectively. Western blot data showed that ALDH2-siRNA downregulated the protein expression of ALDH2 by 70% compared with the control and scramble-siRNA group (Figure 2B and 2C; \( P < 0.05 \)). Indeed, comparable regulation of ALDH2 was noted in microvascular genesis in vitro (Figure 2D and 2E). There was no significant difference among control, scramble-siRNA, and ALDH2-siRNA groups under normoxia. After a 6-hour hypoxia, formation of capillary-like tubes by microvascular endothelial cells was increased by 2-fold compared with formation...
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of capillary-like tubes by normoxic cells in both control and scramble-siRNA–transfected groups. Interestingly, the hypoxia-induced tube formation of endothelial cells seemed to be negated in ALDH2-siRNA–transfected group (P < 0.05; Figure 2D and 2E). The angiogenesis process is also committed to proliferation and migration of endothelial cells. Wound healing assay was performed to evaluate the role of ALDH2 in endothelium migration. To get long-term ALDH2-knockdown and ALDH2-overexpression cell line, and also to reduce mouse euthanization, the following experiment was performed in human umbilical vein endothelial cells. The above row showed the wound at 0 hour, and the below row showed the wound at 6 hour after normoxia or hypoxia (Figure 2F). The average migration distance was measured. Quantitatively,
The result showed the same pattern as in the wound healing model. ALDH2-regulated angiogenesis, the HIF-1α/VEGF pathway was evaluated. Control, ALDH2 gain- and loss-function cell models were established using lentiviral vectors to determine the role of ALDH2 in this process. Cells were exposed to normoxia or hypoxia for 6 hours before assessment of protein levels for HIF-1α and VEGF. The immunoblot analysis depicted a significant increase in the levels of HIF-1α and VEGF in the control group after 6-hour hypoxia, the effect of which was abolished in ALDH2-KO group (P<0.05; Figure 3A and 3C). In contrary, the HIF-1α and VEGF expression showed a significant increase in the ALDH2-transfected group under normoxia. Quantitatively, there is a 2-fold increase in the levels of HIF-1α and VEGF in the ALDH2-transfected group compared with the control normoxic group (equivalent to the control-hypoxia group). After 6-hour hypoxia, the HIF-1α and VEGF expression was increased slightly in ALDH2 overexpression hypoxic group compared with ALDH2 overexpression normoxia group (Figure 3E, 3G, and 3H). Taken together, these data indicate that ALDH2 is required in HIF-1α/VEGF pathway and that the regulation seemed to be hypoxia independently in vitro.

To investigate whether ALDH2 regulates HIF-1α/VEGF signaling in vivo, expression of these proteins was evaluated in ischemic quadriceps from both nonischemic and ischemic limbs in WT and ALDH2-KO mice, on days 1, 7, and 21 after hindlimb surgery (Figure 4A). In this immunoblot analysis, the nonischemia quadriceps of each mouse was used as control. The data revealed that there was no significant increase of HIF-1α/VEGF in WT mice on day 1 after hindlimb ischemia (Figure 4B, 4D, and 4E). However, ALDH2-KO showed temporary increase of HIF-1α (≈2-fold; P<0.05; Figure 4B and 4D) and VEGF (≈1.5 fold; P<0.05; Figure 4B and 4E). However, interestingly, although there was significant increase of HIF-1α (≈1.2 fold; P<0.05; Figure 4F and 4H) and VEGF (≈1.5 fold; P<0.05; Figure 4F and 4I) in WT mice at the day 7 after surgery, the ALDH2-KO mice showed few accumulation of HIF-1α and VEGF (Figure 4F, 4H, and 4I). Finally, the expression of HIF-1α and VEGF was comparable between WT and ALDH2-KO groups on day 21 (Figure 4J, 4L, and 4M).

ALDH2 Regulates HIF-1α and VEGF Expression In Vitro and In Vivo

Figure 3. Regulation of aldehyde dehydrogenase 2 (ALDH2) on hypoxia-inducible factor (HIF)-1α and vascular endothelial growth factor (VEGF) signaling pathway in vitro. A, Representative Western blot of VEGF, HIF-1α, and ALDH2 in human umbilical vein endothelial cells (HUVECs) with negative control or ALDH2-shRNA lentiviral transfection, cultured in normoxia and 6 hours of hypoxia. B, Quantitative analysis of ALDH2 expression in ALDH2-shRNA lentiviral-transfected cells. C and D, Quantitative analysis of HIF-1α and VEGF expression after hypoxia treatment. E, Representative Western blot of VEGF, HIF-1α, and ALDH2 in HUVECs with negative control or ALDH2-cDNA lentiviral transfection, cultured in normoxia and 6 hours of hypoxia. F, Quantitative analysis of ALDH2 expression in ALDH2-cDNA group. G and H, Quantitative analysis of HIF-1α and VEGF expression in ALDH2 overexpression ion cells. Mean±SEM, n=3 to 5, *P<0.05. WT indicates wild-type.
that PHD2, as a cellular oxygen sensor, may regulate HIF-1α protein level through promoting its degradation. Therefore, PHD2 expression was explored in our study (Figure 4B, 4F, 4G, 4K, 4L, and 4P). However, we did not find any difference in PHD2 protein level among the 4 experimental groups at days 1, 7, and 21, in vivo. These data demonstrated that although ALDH2-KO promoted HIF-1α/VEGF expression transiently early on, it later downregulated the level of HIF-1α/VEGF, which was PHD2 independently, eventually leading to impaired angiogenesis, revascularization, tissue repair, and preservation of function.

ALDH2 Influences the Mobilization But Not the Planting and Repair Effects of Endogenous Stem/Progenitor Cells

Revascularization of ischemic injury is thought to be driven, in part, by the bone marrow stem or progenitor cells. We therefore investigated the effects of ALDH2-KO on endogenous stem/progenitor cells repair and whether such effects contributes to the difference of revascularization between WT and ALDH2-KO mice. First, the mobilization of EPC was measured by flow cytometry at day 7 after hindlimb ischemia, shown as the ratio of CD34+/Flk1+ cells to mononuclear cells in peripheral blood, and the ratio of CD34+/Flk1+ cells to bone marrow cells in bone marrow, of both WT and ALDH2-KO mice. Interestingly, circulating EPC of KO mice was significantly increased when compared with WT mice (Figure 5A; \( P<0.05 \)), and the bone marrow EPC was slightly decreased in KO group (Figure 5B; NS). We speculated that the enhanced mobilization of EPC in ALDH2-KO mice is a kind of compensatory response to the worse blood supply in ischemic hindlimb. To further test the role of ALDH2 in post-ischemia endogenous stem/progenitor cells repair, we next performed the cross bone marrow transplantation between WT and ALDH2-KO mice (protocol was shown as Figure 5C). Assessment of blood perfusion recovery by laser Doppler at day 21 after hindlimb ischemia showed that KO mice with WT bone marrow cells (W2K) displayed worse perfusion than WT mice with WT bone marrow cells (W2W), and KO mice with KO bone marrow cells (K2K) displayed worse perfusion than WT mice with KO bone marrow cells (W2W). There was no difference between WT mice with KO bone marrow and WT mice with WT bone marrow, or between KO mice with KO bone marrow and KO mice with WT bone marrow (Figure 5D and 5E). Collectively, these data indicated that genotype of recipient was a major determinant affecting the blood perfusion recovery, but not the genotype of bone marrow donor. We also observed the recruitment and planting of bone marrow–derived EPCs to ischemic area by immunofluorescence stain (protocol was shown as Figure 5C). Before the transplantation, bone marrow stem/progenitor
cells purified from WT and ALDH2-KO mice were exposed to GFP-lentiviral for 12 hours, which enabled at least 50% of bone marrow cells to express GFP continuously. Therefore, GFP can be used as a marker for bone marrow-derived cells. Immunostaining of tissue from ischemic muscles showed that there was no significant difference on the number of GFP+/CD31+ cells among the 4 groups, indicating that equivalent level of bone marrow-derived EPC was planted in the ischemic area of these 4 groups, eventually. Taken together, these data demonstrated that although ALDH2-KO mice mobilized more EPC after hindlimb ischemia, the same level of bone marrow-derived EPC was found in the ischemic area, which might be caused by the worse ischemic micro-environments in KO mice. The blood perfusion recovery seemed mostly dependent on the ALDH2 genotype of recipient but not of the bone marrow. Therefore, endogenous stem/progenitor cell repair was barely the reason of the impaired postischemia revascularization in case of ALDH2 deficiency.

Figure 5. Repair of endogenous stem/progenitor cells in bone marrow transplantation mice. A, Quantitative analysis of circulating endothelial progenitor cells (EPCs) at day 7 after hindlimb ischemia surgery. B, Quantitative analysis of bone marrow EPC at day 7 after hindlimb ischemia surgery; in (A) and (B) EPC were measured by flow cytometry displayed as the ratio of CD34+/Flk1+ cells to mononuclear cells or bone marrow cells, respectively. C, Experimental protocol. D, Original Laser Doppler perfusion images displaying hindlimb perfusion 21 days after ischemia surgery in mice performed cross bone marrow transplantation. E, Perfusion recovery in mice performed cross bone marrow transplantation. F, Representative original micrographs of hindlimb sections 21 days after surgery; CD31 (+) stained are shown in red; GFP (+) cells are labeled as bone marrow-derived cells in blue. G, Quantitative analysis of bone marrow-derived endothelium cells (number of CD31 (+) and GFP (+) cells normalized by the ratio of GFP positive rate cells in bone marrow). Mean±SEM, n=3 to 5, *P<0.05. ALDH indicates aldehyde dehydrogenase; BMC, bone marrow cell; DAPI, 4′,6-diamidino-2-phenylindole; KO, knockout; and WT, wild-type.
**ALDH2 Glu504Lys Polymorphism Contributes to Poor CCC**

The correlation between development of CCC and ALDH2 polymorphism was evaluated in a subset of Chinese patients. As described, collateral circulation by coronary angiography from 139 patients with CTO were examined. The patients were divided into 2 groups, namely poor and enriched CCC (Table 1). There were little differences between the 2 groups with regard to sex, age, left anterior descending coronary artery, right coronary artery, type 2 diabetes mellitus, hypertension, smoking, drinking, and hyperlipidemia (Table 1). Of the 139 patients, 51 (34.09%) patients had poor CCC and 88 (63.31%) displayed enriched CCC. Our result revealed that ALDH2 AA genotype (mutant homozygotes) exhibited a higher frequency in the poor group than in the enriched group (Table 2; 11.76% versus 1.14%; \( P=0.01 \)), using a 2-tailed Fisher exact test. Furthermore, the ALDH2 AA homozygotes displayed a significant lower rate in enriched CCC than those carrying at least 1 functional ALDH2*1 allele (WT allele; 14.29% versus 65.91%), resulting in an odds ratio of 0.08 (95% confidence interval, 0.009–0.701; \( P=0.026 \)) in the logistic regression model. A marginal \( P \) value was detected in the association between time duration and CCC, it appeared that longer duration was positively correlated with good CCC (Table 1; 82.35% versus 93.18%; \( P=0.047 \)). This variant was added into the logistic regression analysis, resulting no significant effect (\( P>0.05 \)). Therefore, these findings depict that loss-of-function ALDH2 mutation contributes to risk of poor CCC in patients with CTO independently. To recapitulate our laboratory finding in the clinic setting, we further assessed the correlation between the ALDH2 polymorphism and the serum VEGF levels in patients with CTO. Our result revealed that serum VEGF levels displayed a non-significant increase in the enriched CCC group compared with those in the poor CCC group (Figure 6A; 256.4±37.01 versus 233.3±27.79; \( P=0.618 \)). As expected, patients with ALDH2 mutation displayed a trend of lowered serum VEGF levels compared with patients with WT ALDH2 phenotype (Figure 6A; 209.9±20.98 versus 279.5±42.62; \( P=0.146 \)). These clinical data, therefore, indicated that there may be a correlation between ALDH2 mutation and poor CCC possibly resulted from reduced serum VEGF levels in patients with CTO (Table 3).

**Discussion**

The salient findings from this study revealed that global ALDH2 deficiency led to unfavorable vascular sequelae including worsened blood supply after ligation of femoral artery accompanied with fewer numbers of capillary and small arteries, overt muscular atrophy, and reduced capacity of endothelial cells to proliferation, migration, and form tube-like structure. Our data further suggested that the loss of ALDH2-regulated essential angiogenesis factors such as HIF-1α/VEGF pathway may underscore such anomalies. Results from bone marrow transplantation showed that the impaired postschemia repair because of ALDH2 deletion was not related to endogenous stem/progenitor cells. Clinical observation provided compelling evidence in that patients with ALDH2 deficiency were more vulnerable to poor CCC development possibly associated with reduced serum VEGF levels in patients with CTO. Our study demonstrated, for the first time, that ALDH2 possesses an intrinsic capacity to regulate ischemia-hypoxia-induced angiogenesis, involved in the ischemic microenvironment homeostasis and organ function preservation, via HIF-1α/VEGF cascade, and therefore, the common ALDH2 Glu504Lys mutation exhibit a great clinical impact on the fate of CCC in patients with chronic heart ischemia.

Chronically ischemic tissue, a hallmark for peripheral and coronary artery disease, requires the remodeling from vascular network to reconstitute and sustain its viability. Therefore, therapeutic angiogenesis remains an unmet medical need. However, a tremendous interindividual variability exists in the degree of new collateral formation in patients with coronary artery disease. The observed interindividual heterogeneity may be because of environmental, genetic and epigenetic causes, complications or the severity and duration of disease. Still, the mechanism underneath this interindividual heterogeneity remains poorly understood. Among various potential contributing factors, genetic mutations not only affect the ischemia-angiogenesis response process but also limit the efficiency of revascularization promote therapeutics. To this end, genetic predisposition may be considered as, perhaps, one of the major host factors crucial for the reestablishment of functional collateral circulations. Coronary CTO is considered to be the last frontier of coronary interventional medical specialties. The formation of CCC in patients with CTO is essential to the maintenance of cardiac function.

This study showed an inverse correlation between ALDH2 rs671 polymorphism and CCC formation in patients with

<table>
<thead>
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<th>Parameters</th>
<th>Poor Enriched ( P ) Value</th>
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<tbody>
<tr>
<td>Sex</td>
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<tr>
<td>Male</td>
<td>41 (80.39%) 79 (89.77%)</td>
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<tr>
<td>Female</td>
<td>10 (19.61%) 9 (10.23%)</td>
</tr>
<tr>
<td>Age</td>
<td>56.6±7.9 58.1±7.9</td>
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<td>Duration</td>
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<tr>
<td>≤3 mo</td>
<td>9 (17.65%) 6 (6.82%)</td>
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<tr>
<td>&gt;3 mo</td>
<td>42 (82.35%) 82 (93.18%)</td>
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<tr>
<td>Risk factors</td>
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<tr>
<td>Diabetes mellitus</td>
<td>17 (33.33%) 24 (27.59%)</td>
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<tr>
<td>Hypertension</td>
<td>27 (52.94%) 44 (50.00%)</td>
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<tr>
<td>Drinking</td>
<td>12 (24.49%) 20 (24.20%)</td>
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<td>Smoking</td>
<td>21 (42.00%) 39 (44.32%)</td>
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<td>TG</td>
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<tr>
<td>HDL</td>
<td>0.99±0.24 1.04±0.26</td>
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<tr>
<td>LDL</td>
<td>2.24±1.00 2.32±1.16</td>
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Mean±SEM. CCC indicates coronary collateral circulation; HDL, high density lipoprotein; LDL, low density lipoprotein; TC, total cholesterol; and TG, total triglyceride.
induced myocardial anomalies through reduced myocardial apoptosis. These data showed significant protective function. This piece of evidence implied that ALDH2 may be protective for cardiomyocyte through enhancing homeostasis of microenvironment under ischemic conditions via promoting revascularization and perfusion.

Angiogenesis is the process through which new blood vessels form from pre-existing vessels. This is distinct from vasulogenesis, and is responsible for majority of blood vessel growth during development and in pathological conditions. A hypoxia-inducible program, driven by HIF-1α, renders endothelial cells responsive to angiogenic signals. Normally, when sufficient oxygen is available, HIF-1α may be hydroxylated by the oxygen-sensing enzymes named proteins PHD1–3. And the hydroxylated HIF proteins would next be degraded by proteasomal degradation. Although hypoxia inactive the PHDs, the upregulation of HIF-1 correlates with its combination to the hypoxia response element of its extensive target genes, mainly responsible for oxygen supply and cell survival, such as VEGF, then subsequently initiates the process of angiogenesis. Therefore, the use of HIF-1α inhibitors to block tumor or ocular angiogenesis has received attention, and conversely, HIF-1α gene transfer in mice or activation of HIF-1α promotes ischemic tissue revascularization. In our study, ALDH2 was found to regulate hypoxia-/ischemia-induced HIF-1α and then accumulation of its downstream angiogenic protein VEGF. However, this regulation was independent of PHD2. It seems that HIF-1α is not regulated only by the oxygen tension but also by various other stimuli, such as transition metals, nitric oxide, reactive oxygen species, growth factors, and mechanical stresses and the activation of signal proteins, such as p53, Akt, mTOR, MEK1/2, Erk1/2, and so on.

Significantly, ALDH2 activity would be strongly affected by genetic mutation. For instance, the common human polymorphism in ALDH2, in which glutamate at amino acid 504 is replaced by lysine, displays a dramatically high prevalence (~40%) in Asian populations. It is well conceived that ALDH2 acts as a homotetramer or heterotetramer, and all tetramers that contain at least 1 ALDH2*2 subunit being inactive. It was well conceived that ALDH2 acts as a homotetramer or heterotetramer, and all tetramers that contain at least 1 ALDH2*2 subunit being inactive. Therefore, identification of its clinical phenotype is rather important to the health impact of ALDH2 gene. This interindividual heterogeneity may also help to explain the variable angiogenic responses seen in other conditions, such as diabetic retinopathy and solid tumors.

In summary, ALDH2 deficiency compromises regenerative capacity of ischemic tissues as a result of impaired

Table 2. Genotypes and CCC

<table>
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<th>Parameters</th>
<th>Poor (%)</th>
<th>Enriched (%)</th>
<th>P Value</th>
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<td></td>
<td></td>
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<tr>
<td>AA</td>
<td>6 (11.76)</td>
<td>1 (1.14)</td>
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<tr>
<td>GA</td>
<td>19 (37.25)</td>
<td>33 (37.50)</td>
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<tr>
<td>GG</td>
<td>26 (50.98)</td>
<td>54 (61.36)</td>
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Mean±SEM. A indicates ALDH2 mutant type; ALDH, aldehyde dehydrogenase; CCC, coronary collateral circulation; and G, ALDH2 wild-type.

Table 3. Logistic Regression

<table>
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<th>Effect</th>
<th>Odds Ratio Estimates</th>
<th>95% Wald Confidence Limits</th>
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<td>Genotype AA vs GG</td>
<td>0.08</td>
<td>0.009</td>
<td>0.701</td>
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<tr>
<td>Genotype GA vs GG</td>
<td>0.836</td>
<td>0.402</td>
<td>1.741</td>
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Mean±SEM. A indicates ALDH2 mutant type; ALDH, aldehyde dehydrogenase; and G, ALDH2 wild-type.

Figure 6. Correlation of aldehyde dehydrogenase 2 (ALDH2) Glu504Lys polymorphism with coronary collateral circulation and serum vascular endothelial growth factor (VEGF). A, Serum VEGF level of patients with enriched or poor CCVs. B, Serum VEGF level of patients with wild-type or mutant ALDH2. Mean±SEM, n=139.
angiogenesis via an HIF-1α-/VEGF-dependent mechanism. Moreover, ALDH2 mutation displayed an inverse correlation coronary collateral vessel formation in patients. This effect is of significant clinical implication as loss-of-function for ALDH2 represents the most popular human genetic mutation. Our findings offer compelling evidence to support that AA genotype may require more aggressive therapeutic attention for vascular recanalization. Last but not least, our investigation revealed therapeutic potential for ALDH2 in the revascularization therapy in patients with coronary heart disease.

Limitation

One obvious limitation noted for the clinical study is that we had little information of the blood supply in patients with CTO before the total occlusion period. Poor blood supply frequently occurs before coronary occlusion and might contribute to the poor CCC.

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Disclosures

None.

References


Our work described a novel finding that aldehyde dehydrogenase 2 regulates the angiogenesis and revascularization induced by chronic ischemic injury in both mice and human subjects. This study is of particular clinical importance. First, this study may advance our understanding in that genetic variant serves as a hereditary regulatory factor in angiogenesis. Furthermore, it provides experimental evidence, which explains possible heterogeneous clinical manifestation and outcomes in certain chronic ischemic disease. Finally, given the large portion of aldehyde dehydrogenase 2 mutant carrier and the high prevalence of chronic ischemic disease, this study should shed some lights in individualized revascularization therapy.
Mitochondrial Aldehyde Dehydrogenase 2 Regulates Revascularization in Chronic Ischemia: Potential Impact on the Development of Coronary Collateral Circulation

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Materials and Methods

1. Animals
   Male and female C57BL/6 mice (8 weeks old, weighing 20g-22g) were obtained from the Shanghai Animal Administration Centre (Shanghai, China). Age- and weight-matched ALDH2 knockout mice were generated as described previously. Mice were housed at room temperature under a 12/12 light/dark cycle with free access to water and standard laboratory mouse chow. Adult male Wistar rats weighting 200±10g were obtained from the Shanghai Animal Administration Centre too, were used for rat cardiac microvascular endothelial cells (RCMECs) culture, and operated as described. Animal experiment protocols were approved by the Animal Care and Use Committee of Fudan University. All experiments were performed according to the guidelines of “the Guide for the Care and Use of Laboratory Animals” Academy Press (NIH Publication No. 85–23, revised 1996).

2. Hind-Limb Ischemia Model
   Mice were anaesthetized with a single intraperitoneal ketamine injection (80μg/g body weight). Chronic ischemia was generated in compliance with well-established unilateral hind-limb surgery protocols. In brief, following a 1 cm skin incision was made at the medial thigh; the femoral artery was separated from the femoral vein and nerve. The part proximal to the outlet of the profunda femoris artery and the distal end (outlet of the saphenous artery) were ligated with 5–0 silk sutures. The femoral artery was then excised between the ligations. Wound closures were conducted with single-layer sutures with 4–0 Prolene threads.

3. Laser Doppler Perfusion Imaging
   Limb perfusion was assessed by laser Doppler perfusion imaging (PeriScan PIM 3 system, Perimed, Sweden). Before the assay, anesthetized mice were placed on a heating pad at 37℃ for 5 min to maintain body temperature. Perfusion was assessed on day 1, 14 and 21 after the surgery. Perfusion ratio of ischemic limb versus non-ischemic hind-limb was quantified by averaging relative units of flux from knee to toe using PIMsoft Software (Perimed med, Sweden).

4. Immunofluorescence (IF) Staining
   Paraffin-embedded serial sections (6 um) obtained from skeletal muscles of the
ischemic hind limb of WT and KO mice were used for IF staining. To define small arteries, capillary on day 21 after surgery, a primary antibody against α-smooth muscle actin (Santa Cruz Biotechnology, Santa Cruz, CA) and a primary antibody against CD31 (BD, United States) were used to detect smooth muscle cells and endothelial cells, respectively. Tissues were mounted by the use of Vectashield DAPI nuclear counterstain.

5. Cell Culture, Transfection and Hypoxia Model

Adult male Wistar rats weighting 200±10g were anesthetized by intraperitoneal injection of 50mg/kg sodium thiopental. Under sterile conditions, a midline thoracotomy was performed. The heart was cannulated, the left atrium, right atrium, right ventricular was incised, and the left ventricular were flushed with 20ml of cold PBS. Remove the endocardial and epicardial. Then cut the tissue into 1 mm³ pieces. The tissue was planted in the petri dish and put 2-3ml fetal bovine serum (FBS) then were added another 3-4 ml of DMEM with 20% FBS and then were placed back to incubator. On culture day 72h, cells migrated out from the explants, then removed explants and rinsed gently with the PBS. The passages of cells between 2 and 4 were used in our experiment. HUVEC cell line was obtained from Cell Bank of Chinese Academy of Science (Shanghai, China). For hypoxia treatment, cells were incubated in a hypoxic chamber for 6 hours. ALDH2-siRNA was used to inhibit expression of ALDH2 in MMVECs. Lenti viral vectors (Hanyin Co, shanghai, China), were employed to deliver ALDH2 or to inhibit the expression of ALDH2 in the HUVEC cell line.


Endothelial Tube formulation assay was performed as previously described. Briefly, MMVECs from different groups were seeded on the top of 200 μl pre-polymerization growth factor-reduced Matrigel (BD Biosciences, UK) in 24-well culture plates and exposed to normal or hypoxia for 6 hours. At the end of experiment, floating cells were removed by washing and endothelial networks were counted under an inverted microscope.

Wound healing assay was performed as previously described. Briefly, HUVECs were seeded on 6-well plates grew to full confluence. Subsequently, cells were wounded by pipette tips and washed twice with PBS to remove detached cells, and photomicrographs of initial wounds were taken. After 6 hours hypoxia, photomicrographs of final wounds were taken for each group. Initial and final areas covers by cells were measured using Image J software, and difference between the two was used to determine migration distance using the formula: Final cell area minus initial cell area, then divided by the width.

CCK-8 assay was performed using Cell Counting Kit-8 (Dojindo Laboratories, Japan).
7. Immunoblot Analysis

Immunoblot analysis was carried out as described previously. Proteins were separated on 12% SDS-polyacrylamide gel, transferred to polyvinylidene fluoride membranes, and subsequently subjected to immunoblot analysis using appropriate antibodies. Anti-ALDH2 antibody (1:2000) was purchased from Epitomics Inc. (Burlingame, CA 94010-1303 U.S.A.). The anti-HIF-1α antibody (1:1000) was from NOVUS Biological Inc. (Littleton, CO 80120 U.S.A.) and the anti-VEGF antibody (1:2000) from Abcam Inc. (Cambridge, MA 02139-1517 U.S.A.). Immunoreactive antigen was then detected using enhanced chemiluminescence detection.

8. Flow Cytometry Measurement

To assess the percentage of endothelial progenitor cells those were mobilized to the circulation after hind-limb ischemia surgery, blood samples and bone marrow were harvested from animals 7 days after injury. Red blood cells were lysed at room temperature for 5 minutes with erythrocyte lysis buffer, followed by washing with PBS. Cells were fixed with 4% paraformaldehyde and blocked with 1% BSA in PBS followed by staining with antibodies, APC labeled antibody against Flk-1 and FITC labeled antibody against CD34 (both were purchased from BD, United States) for 30 minutes at 4 degree, then washed 3 times with PBS. Cells were analyzed on a FACScan flow cytometer (Becton Dickinson, Franklin Lakes, NJ).

9. Bone Marrow Transplantation

Wild-type C57BL/6J and ALDH2-KO mice were sacrificed to harvest bone marrow cells. Lineage-/- bone marrow cells were obtained using flow cytometry sorting, then transfected with GFP-lentiviral. Wild-type C57BL/6J and ALDH2-KO mice were lethally irradiated (8 Gy). On the same day, those mice were injected with 1*10^6 GFP labeled lin-/- bone marrow cells. Reconstitution of bone marrow was determined by flow cytometry 1 month after transplantation. The average percent reconstitution was determined to be between 90% and 95%, with about 30-50% GFP–positive cells in the marrow 4 weeks after transplantation.

10. Patients

From October 2011 to April 2014, consecutive patients with angiographically documented CTO were included using the inclusion criteria of 1) displaying CTO in at least 1 major epicardial coronary artery (left main coronary artery, left anterior descending artery or left circumflex artery), 2) as TIMI flow 0 for more than 6 weeks; by the exclusion criteria of 1) undergoing acute myocardial infarction within 90 days, 2) previous percutaneous coronary intervention or coronary artery bypass grafting, 3) congenital heart diseases or valvular heart diseases, or over 70 years old. A total of 139 patients were recruited to make up the study cohort. The study protocol was approved by the ethical review of Zhongshan Hospital, Fudan University, and all patients provided written informed consent to participate in the study.

11. Patient Data Collection
For each patient, a data sheet was completed with the patient’s name, identification number, age, sex, previous revascularizations, past medical history of hypertension, diabetes, cigarette smoking, and HLP (hyperlipidemia). Coronary angiography was performed by the use of standard techniques. CTO was defined by the presence of a coronary artery stenosis resulting in complete interruption of antegrade flow in a major epicardial coronary artery or minimal contrast penetration through the lesion without distal vessel opacification. Retrograde collateral filling of the vessel distal to a CTO was assessed by experienced interventional cardiologists blinded to other clinical and imaging data. The diameter and angiographic flow of collateral vessels was semiquantitatively assessed by the use of the Rentrop classification (class 0 = no visible filling of collaterals, class 1 = filling of side branches, class 2 = partial filling of epicardial segment of the occluded vessel, class 3 = total filling of epicardial segment). In the current study the grade 0 or 1 were taken as poor CCC and grade 2 or 3 as rich CCC.

12. Genotyping
Genomic DNA was extracted from leukocytes, and the DNA sequence spanning the ALDH2 polymorphism was amplified by PCR. Genotyping was performed by direct sequencing, using an ABI GeneScanTM-500 (Genesky Biotechnologies Inc., Shanghai, China).

13. Statistical Analysis
The continuous variables were expressed as the means ± SEM and compared with Student’s independent t test to analyze 2 groups or one-way ANOVA to analyze multiple groups. The categorical variables were expressed as percentages, and the χ² test was applied for the determination of significance of an association. Lab data were analyzed with GraphPad Prism 5 and SPSS version 16.0. In clinical study the effect of the covariants was examined by multiple logistic regression with SAS version 9.2. The value of P < 0.05 was considered statistically significant.


