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Aerobically Derived Lactate Stimulates Revascularization and Tissue Repair *via* Redox Mechanisms

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ABSTRACT

Hypoxia serves as a physiologic cue to drive an angiogenic response *via* HIF-dependent mechanisms. Interestingly, minor elevation of lactate levels in the tissue produces the same effect under aerobic conditions. Aerobic glycolysis contributes to lactate accumulation in the presence of oxygen, especially under inflammatory conditions. We previously postulated that aerobic lactate accumulation, already known to stimulate collagen deposition, will also stimulate angiogenesis. If substantiated, this concept would advance understanding of wound healing and aerobic angiogenesis because lactate accumulation has many aerobic sources. In this study, Matrigel plugs containing a powdered, hydrolyzable lactate polymer were implanted into the subcutaneous space of mice. Lactate monomer concentrations in the implant were consistent with wound levels for more than 11 days. They induced little inflammation but considerable VEGF production and were highly angiogenic, as opposed to controls. Arterial hypoxia abrogated angiogenesis. Furthermore, inhibition of lactate dehydrogenase by using oxamate also prevented the angiogenic effects of lactate. Lactate monomer, at concentrations found in cutaneous wounds, stabilized HIF-1α and increased VEGF levels in aerobically cultured human endothelial cells. Accumulated lactate, therefore, appears to convey the impression of "metabolic need" for vascularization, even in well-oxygenated and pH-neutral conditions. Lactate and oxygen together stimulate angiogenesis and matrix deposition. *Antioxid. Redox Signal.* 9, 1115–1124.

INTRODUCTION

Hypoxia, caused by disrupted vasculature, peripheral vasculopathies, or pulmonary disorders, is a key factor that limits dermal wound healing (16, 18, 23). A threshold level of oxygenation is required for tissue remodeling. Hypoxia can initiate neovascularization by inducing growth factors (12, 19, 36, 39). However, sustained periods of equally severe hypoxia limit neovascularization (17), proliferation of dermal cells (28), collagen deposition (33), bacterial killing and resistance to infection (3), and more-severe hypoxia causes tissue death and dysfunction (33). Conversely, supplemental O_2 *in vivo* accelerates wound vessel growth (12, 16, 17), collagen deposition (34, 41), and angiogenesis (12). Intermittent hyperbaric oxygen stimulates vessel growth (17), accelerates the proliferation of granulation tissue (23, 24), and facilitates epithelial healing (34). It also stimulates VEGF, a major long-term angiogenic stimulus at the wound site. Others and we have noted that O_2 treatment induces VEGF gene expression (29) and increases VEGF protein expression in wounds (40). These apparently contradictory data must be understood.

Contrary to views held for almost a century, it is now agreed that most lactate is produced under aerobic conditions, and little correlation exists between lactate and oxygen until the pO_2

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falls to less than ~1 mm Hg (15). Tissue lactate can accumulate to high levels even under conditions of oxygen sufficiency, particularly in wounds where, regardless of pO_2, lactate increases to the range of 5–15 m*M*, as opposed to the 1–3 m*M* found normally in blood and most uninjured, resting tissues (13). Furthermore, lactate is now understood to have many fundamental metabolic and signaling functions (15).

Angiogenesis may be viewed as a long-term response to unmet metabolic need. It is well demonstrated that hypoxia or dysoxia or both are master controllers of the signals that drive new vessel formation (37). Lactate is also a known instigator of cytokines and growth factors such as VEGF, TGF-β, and IL-1 (10, 41). Lactate stabilizes HIF-1α, even in the presence of oxygen, because lactate and pyruvate bind to and inhibit the HIF prolyl hydroxylases that would otherwise hydroxylate HIF-1α and mark it for rapid degradation (21, 22). Thus, lactate accumulation, often associated with, but not necessarily a consequence of hypoxia, is poised to express impending hypoxia while not necessarily incurring the hazards of hypoxia itself. It may, therefore, be a more sensitive stimulus for angiogenesis than hypoxia.

In this investigation, we explored the significance of lactate accumulation in two putative roles: (a) as a redox-regulating angiogenic agent, and (b) as a means of reducing the NAD+ level and hence ADP ribosylations that regulate gene transcription and posttranslational modification of many proteins. Both influence angiogenesis and connective tissue deposition (35, 44). The primary goals of this study were to test the central hypothesis that accumulated lactate ion, no matter how derived, (a) is sufficient to induce significant angiogenesis if a physiologic concentration of oxygen is present, and (b) acts *via* redox and polyADP-ribosylation mechanisms. If this hypothesis can be defended, the implication will be that lactate accumulation in the presence of oxygen is a relatively common circumstance and may drive new vessel formation in many aerobic circumstances.

EXPERIMENTAL APPROACH

Lactate delivery

Testing of the central hypotheses *in vivo* required development of an isolated site in tissue in which high, sustained levels of exogenous lactate ion can be achieved by injection without stimulating endogenous production [*e.g.*, *via* inflammation (8)] and where the pO_2 can be independently controlled. To meet these requirements, we devised a Matrigel implant model that shows no ability by itself to induce neovascularization but supports new vessel growth when made host to an angiogenic signal (10). A hydrolyzable lactate polymer was identified that, when added to Matrigel, would increase and sustain an increased lactate monomer level in the gel at a concentration relevant to subcutaneous wounds. In preliminary experiments, we identified a poly-DL-lactide-*co*-glycolide (lactide:glycolide, 50:50; mol wt, 40,000–75,000; Cat. no. P2191; Sigma, St Louis, MO) that served the purpose. Matrigel implants supplemented with this lactate-delivery agent are referred to as lactate-supplemented implants. Hypoxia of less than ~1 mm Hg is known to increase lactic acid production and reduce pH (15). Hydrolysis of the said lactide polymer in water, however, released lactate ion that slightly increases pH because of its association with protons in water. When the lactate polymer was placed in wounds in which extracellular fluid could be collected, the level of lactate monomer increased to a concentration relevant to wounds [4–12 m*M* in mice (41)], and no pH change was detected (41). To test whether surface characteristics of the lactide polymer might be inflammatory and hence might stimulate endogenous lactate production, we tested the effects of a larger polymer poly(L-lactide) (mol wt, 85,000–160,000; Cat. no. P1566, Sigma). The larger polymer did not release lactate at levels released by the previously mentioned lactide polymer (lactide/glycolide, 50:50; mol wt, 40,000–75,000; Cat. no. P2191; Sigma). In contrast, smaller

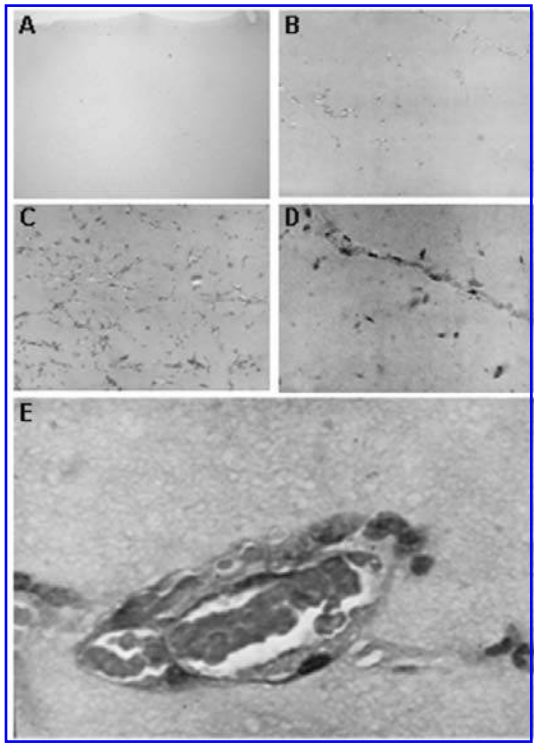

FIG. 1. Grading system used in assessing angiogenesis in Matrigel implants. (**A**) A score of 0 was given for acellular or rare cells. (**B**) A score of 1 reflected scattered endothelial cells in small groups or linear arrangements but without lumens. (**C**) A score of 2 represented endothelial cells in all quadrants of the section, prominent linear arrangements, and some tube formation. (**D**) A score of 3 was assigned for easily identified capillary tube formation, many containing red blood cells and small amounts of collagen. (**E**) A score of 4 was reserved for larger vessels that accommodated more than four red cells abreast and multilayered vessels containing layers of collagen in vessel walls. Agreement between graders was >90%. In no case was the score difference >1 for the same observation by two different graders. The average of three repeated observations by the same graders was noted as the recordable score. Reproduced with permission from the publisher of Hopf *et al.* (17).

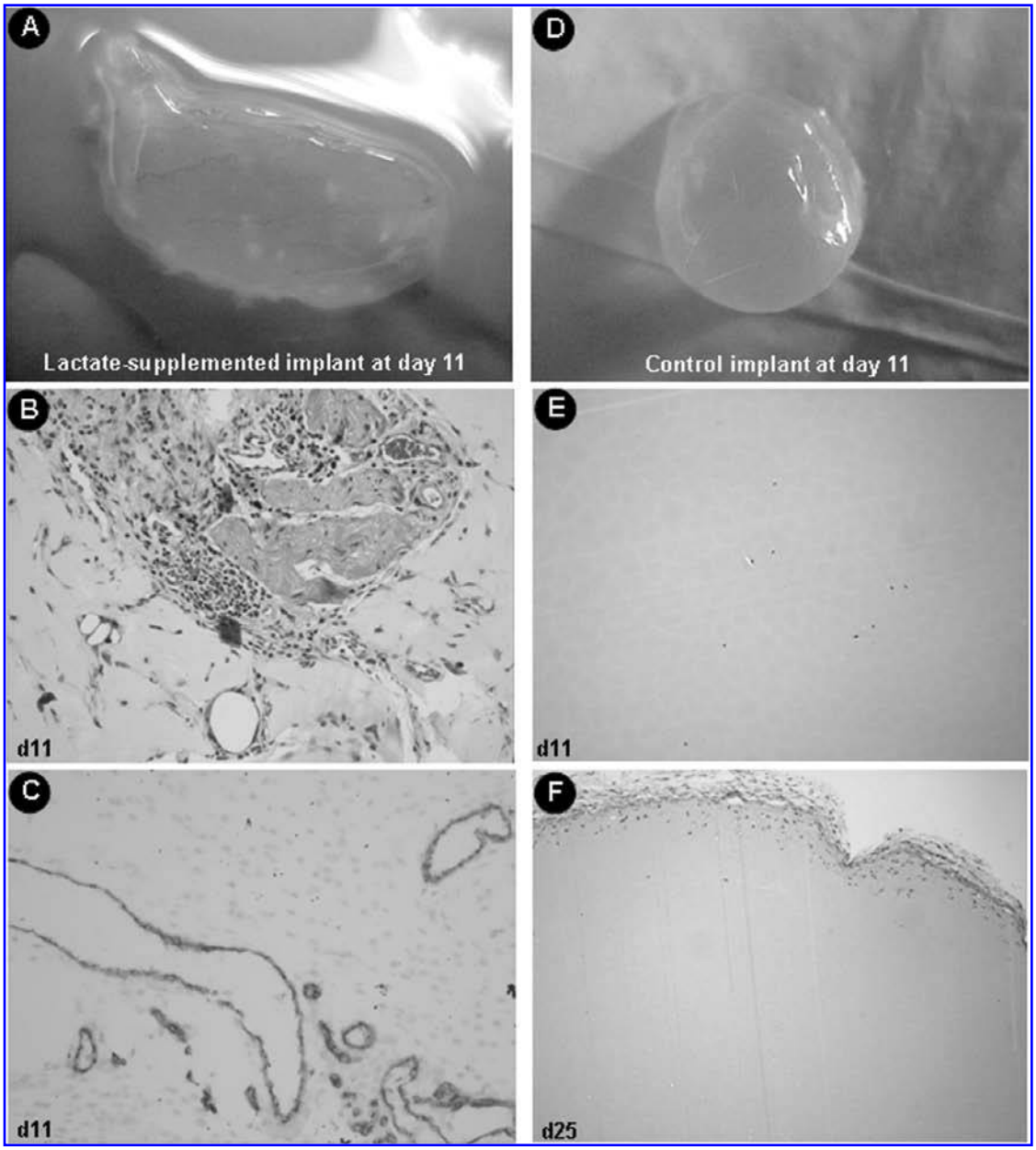

FIG. 2. Blood-vessel growth in lactate-supplemented Matrigel. Matrigel ($n = 80$) containing hydrolysable poly-DL-lactide-*co*-glycolide (lactide/glycolide, 50:50; mol wt, 40,000–75,000), or no additive (control, $n = 100$) were implanted subcutaneously, one injection in each flank just caudal to the ribcage. Four such lactate-supplemented implants were removed and examined microscopically at day 11 or 25 (control), as shown. (**A–C**) Lactate-supplemented (day 11). (**D–F**) Control (D&E, day 11; **F**, day 25). (**A, D**) Intact Matrigel harvest; B&E, hematoxylin & eosin stain. (**C**) CD31 immunostain. (**F**) Hematoxylin & eosin stain on day 25.

polymers induced more rapid but more transient angiogenesis (not shown).

Matrigel implantation and harvest

Six-month-old Swiss Webster female retired breeder mice were used in this study. Powdered lactate polymer was mixed into cold Matrigel (30 mg/ml), and l ml of the mixture was injected subcutaneously in each flank (dorsally, immediately caudal to the ribcage) *via* an 18 needle under isofluorane anesthesia. Controls were similarly injected with control Matrigel. In all, 245 retired breeder mice were injected with 490 implants. The animals were housed in the University of California Laboratory Animal Resource Center. Animals were killed and implants removed at the noted intervals. The institutional committee on animal use approved all procedures before experiments

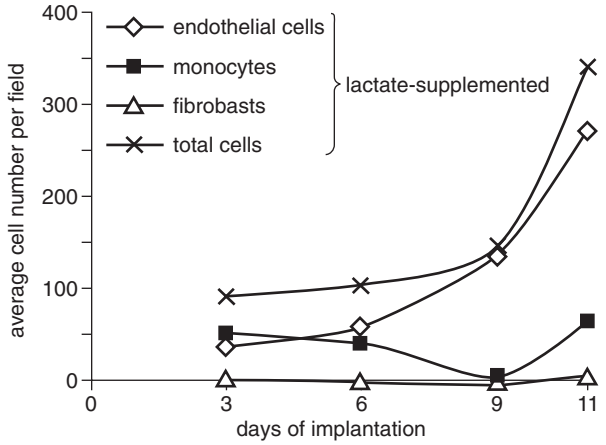

FIG. 3. Kinetics of cellular infiltration in lactate-supplemented Matrigel. Differential cell counts were done on the basis of cellular morphology as identified by hematoxylin and eosin stain. From each implant, two sections were examined. Four fields per slide were examined at 40x magnification, avoiding fields in which the periphery of the implant could be seen. Control slides, from implants either nontreated or treated with high-molecular-weight lactide polymer, showed too few cells to allow differential scoring. Clearly, lactate attracted primarily monocytes and endothelial cells.

were performed. Implants found in (as opposed to on) muscle or the peritoneal cavity (10 of 490 implants) were discarded. On removal, sections were taken from mid implant and were stained with (a) hematoxylin and eosin, (b) purified Rat Anti-mouse CD31 (Pecam-1) monoclonal antibody (BD Biosciences. San Jose, CA), (c) biotin-conjugated Rat Anti-Mouse CD34 monoclonal antibody (BD Biosciences), (d) Rat anti-mouse mac-3 monoclonal antibody (BD Biosciences) to identify macrophages, and (e) Mallory's trichrome (http://stainsfile.info/StainsFile/stain/conektv/tri_mallory.htm) to identify collagen.

Experiment 1. The hypothesis that lactate induces an early invasion of endothelial cells that would be followed by vascularization was tested. The types of cells invading the implants were identified as noted earlier and quantified by averaging the number of each cell type in four high-power (20 × 10) fields of two microscopic sections of each implant and then averaging the number for four implants. It was evident that the changes of interest were most efficiently assessed at 11 days. Sections from day 11 implants were stained with hematoxylin and eosin and Mallory's trichrome stain and were graded for the extent of neovascular formation by two independent and blinded graders. Grading was stepwise as shown in Fig. 1. Note that the day 11 time point was chosen based our examination of sections from days 11, 16, 21, 35, and 50. Results from the time points other than day 11 are not shown.

Experiment 2. The hypothesis that inhibition of lactate dehydrogenase (LDH) C4 with sodium oxamate (27) prevents angiogenesis was examined. This was aimed at expanding on our prior finding that depletion of NAD+ by excess lactate inhibits ADP- and poly(ADPRibosyl)ations and, subsequently, collagen synthesis and deposition (44). An alternate, or more likely, supplementary interpretation would be that conversion of lactate to pyruvate is required for the enhancement of HIF-1α by lactate (21, 22). Sixteen Matrigel implants were placed in eight mice. Eight implants (in four mice) were lactate supplemented and contained sodium oxamate (36 m*M*; Sigma). Eight implants in the remaining four mice contained oxamate but no lactate polymer.

Experiment 3. The hypothesis that the concentrations of lactate and VEGF in the Matrigel implants are quantitatively related was tested. Ten lactate-supplemented and 10 untreated implants were injected into 10 animals on a pair-matched basis. Implants were removed at 11 days and immediately placed in liquid nitrogen. When all implants had been collected, they were quickly thawed, weighed, placed in 0.5 ml saline, and thoroughly macerated, homogenized, and centrifuged. The pellet was used for DNA extraction, and the liquid phase was saved for VEGF analyses, with 0.4 ml of supernatant put aside for the lactate assay (Yellow Springs Instruments Co., Yellow Springs, OH; model 2700 biochemical analyzer with a lactate-sensitive membrane). For VEGF assays, enzyme-linked immunosorbent assay (ELISA) was used (R&D Systems, Minneapolis, MN).

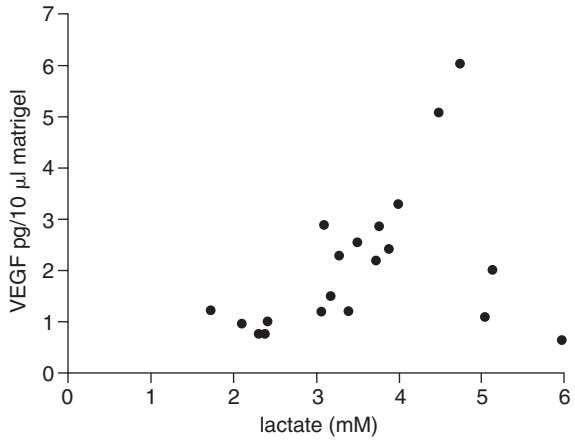

FIG. 4. The relation between lactate and VEGF levels in lactate-supplemented Matrigel. Twenty implants, including both lactate-supplemented and controls, were numbered in ascending order of magnitude of lactate, as shown on the horizontal axis and the squared line. They were then matched with their corresponding VEGF level. When displayed in this manner, VEGF content with respect to lactate at 11 days appears to peak at ~4–5 m*M* lactate. However, the lactate concentrations are somewhat illusory, in that the hydrolysis of the lactate powder is not linear with time. Thus, they can give only an impression of what the concentrations were on days 1–11. Nevertheless, the data support prior experience reported by Beckert (5), who found in cultured endothelial cells that VEGF concentration increased in response to lactate to a peak at ~15 m*M*, and cells deteriorated above that level. This figure, therefore, indicates a significant relation to increasing lactate *in vivo* that reaches a peak and then apparently deteriorates. Lactate levels in controls were 1 m*M*, the same range as found by others in blood and subcutaneous tissues.

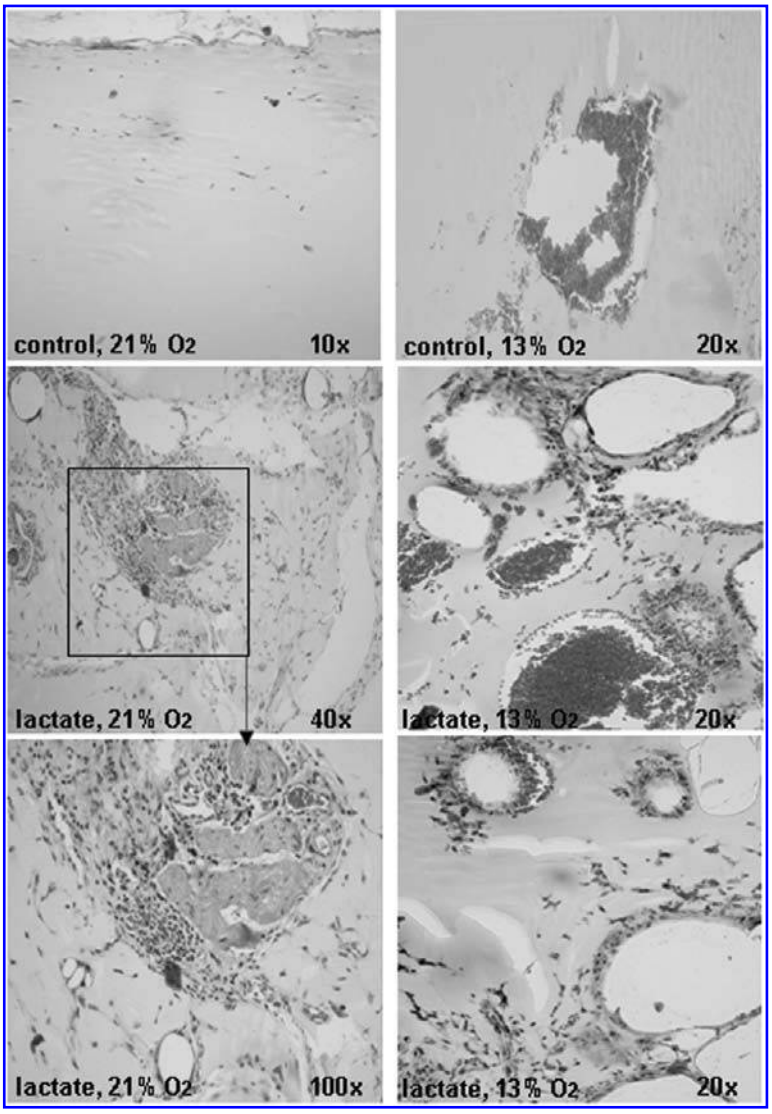

FIG. 5. Histologic characterization of Matrigel implants in normoxic and hypoxic mice. Thirty-six mice were each implanted with lactate-supplemented implant, and one control was not treated. Twelve animals were held in each of three atmospheres: 13% (right column), 21% (left column), and 50% (not shown) oxygen for 11 days. A Pro-ox compact oxygen controller model 110 was used to maintain ambient pO_2 (Reming Bioinstruments, Redfield, NY). FiO_2, humidity (60–80%), CO_2 (<2 mm Hg), and temperature (23–25°C) were maintained within normal limits. Hypoxic (13% O_2 ambience) were housed in a Plexiglas chamber with a single gas inlet and outlet. Air was delivered at 1.0 L/min, and nitrogen, at 4.5 L/min from tanks connected to the chamber by thick-walled polyvinyl tubing. This reliably delivered 13.3 ± 1.3% oxygen to the chamber. Carbon dioxide levels were maintained at 0 mm Hg within the chamber, with a CO_2 absorber (Baralyme, Chemetron Medical Division, St. Louis, MO). A Datex Capnomac II (Datex Medical Instruments, Inc., Tewksbury, MA) was used to sample exhaust gas from the oxygen chambers to confirm the exposure pO_2 and to ensure that CO_2 did not accumulate. Compared with that in normoxic mice (left column), implants in hypoxic mice produced rare and large, thin-walled vessels. These photographs were selected to show as many vessels as possible and are not representative of their abundance. Compared with those in normoxic mice, small amounts of collagen were evident in implants in hypoxic mice. The sections were stained with Masson's trichrome.

Experiment 4. The hypothesis that both oxygen and lactate are necessary for neovascularization was examined. We adopted our previously published approach (17), with the single difference that one of the two implants in each animal was lactate supplemented. Twelve animals were held in each of three atmospheres: 13%, 21%, and 50% oxygen for 11 days continuously, removed only for cage cleaning. The animals tolerated the various oxygen concentrations, showed no signs of discomfort, and lost no weight. Implants were removed at 11 days and stained with both H & E and Mallory's trichrome stain for collagen.

Experiment 5. Finally, we tested the hypothesis that that exposure to lactate increases oxidant production by human endothelial cells. The nitroblue tetrazolium (NBT) test (9) was used for the detection of superoxide anion radical. To test whether lactate-induced superoxide generation is dependent on NADPH oxidases, the flavoprotein inhibitor diphenylene iodonium (DPI) was used.

Statistical analyses

Because the data on histologic scores are step-wise (ordinal), the Kruskal–Wallis statistic and the Mann–Whitney test were used to evaluate differences between groups. Regression analysis was used to assess the correlations between lactate, DNA, and VEGF. Difference between means was examined by Student's *t* test.

RESULTS

On explantation, microscopic sections were examined, as noted in Fig. 1, and grades assigned as described. New vessels could be seen in lactate-supplemented implants with a hand-held (3×) magnifying glass (Fig. 2). Polymer granules could be seen for 30 to almost 50 days. Individual endothelial cells and macrophages in equal numbers were found in all lactate-supplemented implants through 6 days (Fig. 3). Thereafter, in

lactate-supplemented implants, the count of endothelial cells and macrophages increased by three- to fourfold and usually occupied most of each of the implants by days 9 and 11 The numbers of fibroblasts remained unchanged (see Fig. 3). Nonsupplemented implants had too few cells to analyze. In the implants supplemented with the lactate-delivery polymer, VEGF levels were the highest in gel containing 4–5 m*M* lactate ion (Fig. 4). We conclude that a quantitative relation exists between lactate and VEGF. The VEGF response at the higher (>5 m*M*) residual levels probably deteriorated as if at some time, lactate concentrations may have been high enough to injure the cells that produce VEGF during the 11-day exposure.

Every implant of 80 that contained hydrolysable lactate (and were not hypoxic) exhibited neovascular tube formation by 11 days of implantation, with an average score of 2.8 [*i.e.,* most contained linear arrangements and tubules containing erythrocytes (Fig. 5)]. The lowest score in lactate-supplemented implants at day 11 was 1.5 (see Fig. 1 for scoring scale). Relatively few clear examples of true angiogenesis were seen (*i.e.,* invasion of macrophage-led tubes into the implant edge demonstrating development of blood vessels from preexisting vessels). Instead, more evidence was present of isolated endothelial cells and tubes in the Matrigel substance, consistent with *vasculogenesis* (32). By day 11 of implantation, many tubules contained red blood cells, suggesting that hematopoietic stem cells may have differentiated to form functional vessels.

Control implants of the 70 examined, whether loaded with high-molecular-weight polymer ($n = 20$) or containing no polymer ($n = 50$), displayed no appreciable tube formation, and most showed no cellularity, except at the implant edge (see Fig. 2). The highest score was 2, and the average was <1. Therefore, no overlap was noted between control and lactate-supplemented implants ($p < 0.001$). Controls with high-molecular-weight lactate ($n = 25$) were indistinguishable from nontreated controls, except for the presence of polymer granules (not shown). A mild nonspecific inflammation developed around some control implants but migrated into them no farther than ~150 μm from the edge of the implant on day 3. In no such case was any advancement seen after day 6. At 50 days, most of the Matrigel granules had disappeared, but the lactate-rich sites were identified mainly by a vascularized scar. CD31-positive cells were apparent in appreciable numbers at 6 days (not shown). CD31-lined conduits indicative of blood vessel in formation were evident in day 11 (see Fig. 2C). Abundant CD117 (Fig. 6) and CD34-positive cells (not shown) were seen in the lactate-supplemented Matrigel at day 9.

Treatment of Matrigel with the LDH inhibitor oxamate abolished angiogenesis that was evident in lactate-supplemented implants. The average score was 1.0 as opposed to 2.5 in the lactate-supplemented oxamate nontreated implants. No overlap in the scores was found between the two groups, and the difference between the means of the two groups was statistically significant ($p < 0.01$). Thus, LDH activity is required for lactate-induced vasculogenesis.

New vessels were found in all lactate-supplemented implants in air-breathing (Fig. 5, left panel) and hyperoxic (50% O_2, not shown) groups. No angiogenesis score over 1.5 was found in any control implant, whether normoxic, hyperoxic, or hypoxic. The "hypoxic implants" (Fig. 5, right panel shows the characteristic of one of the rare vessels found) showed few new vessels or endothelial cells. Whether lactate-supplemented or control, the few vessels that were seen in hypoxic implants were far larger than noted in the room-air–breathing group, and some contained many red cells (Fig. 5, right panel). The vascular conduits in the implants of hypoxic mice were also immature, poor in collagen, thin-walled, and apparently friable, often ruptured, and resembled cells seen in ascorbic acid depletion when collagen deposition is impaired (6).

Endothelial cells treated with NBT and lactate showed a clear increase in formazan crystals and VEGF production under standard aerobic conditions (Fig. 7). Formazan formation in response to the oxidant-donor SIN-1 demonstrated that the assay used was effective to detect reactive oxygen species. The distribution was, in general, perinuclear. This is consistent with a redistribution or redirection of intracellular oxygen to peroxide production at specific sites, as reported (20). Nonspecific inhibition of the NADPH-linked oxidase by DPI decreased lactate-induced production of superoxides (see Fig. 7). Mean VEGF level in lactate-supplemented cultures was 3.5 times higher than that in cultures not treated with the lactate polymer ($p < 0.01$; Fig. 7). In confirmation of previous reports (20, 22), lactate treatment not only induced VEGF expression but also stabilized HIF-1α expression (Fig. 8).

DISCUSSION

Lactate is unusual among products of glycolysis in that it has many aerobic sources, including exercise, hyperglycemia, lipolysis, sympathetic nervous system activation, and rapid cell division, that characterize wounds and most tumors (15, 17, 21, 22). Leukocytes in particular have limited mitochondrial function when they are activated, as they are in wounds. These cells derive most of their energy from glycolysis, thus releasing large amounts of lactate regardless of the oxygen concentration in the microenvironment (7, 26). Transient inflammation is an obligatory precursor to wound healing. One of the few axioms of wound healing is "no inflammation, no healing." Furthermore, the recent enthusiasm for hypoxia-generated angiogenic signals seems to have obscured the fact that hypoxia impairs collagen synthesis and deposition, both of which are required for angiogenesis (7). No matter how much angiogenic stimulant is present, the functional response cannot proceed in severe hypoxia because new vessels require collagen for strength to withstand the pressures of blood flow. Collagen deposition proceeds only at half its maximal rate at 25 mm Hg pO_2. Furthermore, wounds cannot resist bacterial infection at that level of pO_2 (3). Without collagen, angiogenesis cannot proceed beyond the stage of fragile endothelial buds (7). The problem of decreased collagen deposition in angiogenesis is illustrated by scurvy (ascorbate deficiency), which impairs hydroxylation of collagen, prevents collagen deposition, interferes with angiogenesis, and severely depresses wound healing by producing precisely the same lesion as hypoxia (30). Both hypoxia and ascorbate deficiency block the essential step of collagen deposition in which ascorbate complexes iron, molecular oxygen, and oxoglutarate and hydroxylates-selected proline residues. Without this step, collagen cannot be released from the cell.

Findings of this study demonstrate that relatively minor accumulation of lactate in adequately oxygenated cells and tis-

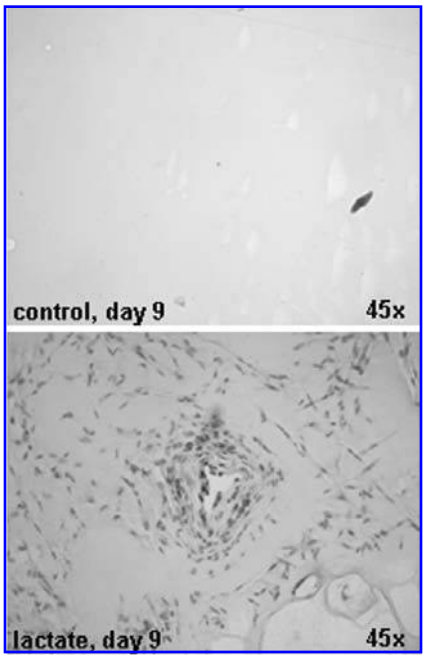

FIG. 6. Recruitment of multipotent hematopoietic stem and progenitor cells in lactate-supplemented Matrigel. CD117 staining is shown. CD117, also known as c-kit, steel factor receptor, and stem cell factor receptor, encodes a 145-kDa cell-surface glycoprotein belonging to the class III receptor tyrosine kinase family. It is expressed on the majority of hematopoietic progenitor cells, including multipotent hematopoietic stem cells as well as committed myeloid, erythroid, and lymphoid precursor cells. In addition to the potential for the differentiation of hematopoietic cells, CD117+ stem cells from murine bone marrow were reported to be capable of differentiation into smooth muscle cells, myocytes, and endothelial cells *in vivo*. CD117 is also expressed on few mature hematopoietic cells (*e.g.*, mast cells). The sections were also richly populated with $CD34^+$ cells (not shown).

sues is itself sufficient to initiate the full sequence of wound healing, including vasculogenesis and collagen deposition. This is, however, only one of many important features of lactate accumulation. Lactate enhances a remarkable set of actions that could be causally linked to its ability to induce the formation of reactive oxygen species. Elevated lactate induces (a) HIF-1α stabilization; (b) VEGF and TGFβ (41); (c) metalloproteinases (25); (d) endothelial cell mobility (5); (e) vascularization (current data); (f) increased collagen synthesis and its posttranslational modification and deposition (13, 41); (g) cell proliferation (43); (h) transcription of genes for proteoglycans, CD44, caveolin-1, Hyal-1 and -2 (11); and (i) an environment suitable for the recruitment of progenitor cells (current data).

The current study supports that whereas hypoxia protects the angiogenic transcription factor HIF-1α from degradation, sustained extreme hypoxia may inhibit angiogenesis. Impairment of angiogenesis by hypoxia in VEGF-supplemented Matrigel has been reported (17). The hypoxia-induced compromise of angiogenesis is coincident with impaired collagen deposition. Consistently, progressive hypoxia has been noted to compromise wound bursting strength (a function of collagen deposition) in colon anastomoses while increasing their VEGF content (4). HIF is first seen in the wound tissue well before pO_2 decreases, whereas lactate levels increase immediately after injury (1). On the contrary, although we observed neither benefit nor inhibitory effects of continuous hyperoxia up to 50% oxygen without added lactate (not shown), Hopf *et al.* (17) showed enhancement of angiogenesis in response to intermittent exposure to 100% O_2 ($pO_2 \cong 180$ mm Hg) (17). Thus, in this context, HIF-1α level and angiogenesis appear to occur in proportion to O_2 and lactate concentrations, with an added factor of time of exposure.

These data fit within a mosaic of oxidant and ADPribosylation mechanisms that are now known to support wound healing. First, it was noted that a lactate:iron chelate produces hydroxyl radical (·OH) in the presence of H_2O_2 by a variant of Fenton chemistry (2). Next, iron-containing structures were observed in the endoplasmic reticulum (ER), and the importance of pO_2 and H_2O_2 that enhanced a Fenton reaction at these sites was reported. H_2O_2 and ·OH were generated in proportion to pO_2. This, in turn, led to translocation of the HIF-1α gene from the ER into the nucleus, elevated HIF-1α, and induced expression of the HIF-1 target genes plasminogen activator inhibitor 1 and heme oxygenase (20). Scavenging of the ·OH attenuated compromise of HIF prolyl hydroxylase activity. They also showed that the increased oxidant flux did not occur in mito-

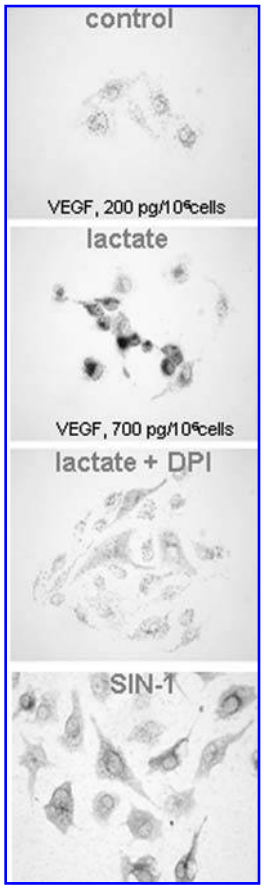

FIG. 7. Lactate-induced oxidant production in microvascular endothelial cells is likely to be NADPH oxidase dependent. Human microvascular endothelial cells (HMVEC-d) purchased from Cambrex Bio Science (Walkersville, MD) and passages five to nine were used for experiments. Cells were grown in endothelial basal medium 2 (CC-3156) containing EGM-2, MV (SingleQuots, Cambrex, Walkersville, MD; CC-4147), 100 units/ml penicillin, 100 units/ml streptomycin, and 0.2 μg/ml amphotericin B. Cultures were maintained at 37°C in humidified 95% air and 5% CO_2 atmosphere. Cells were growth-arrested by incubating overnight in endothelial basal medium 2. Nitroblue tetrazolium (NBT) (1 mg/ml) was added, followed by lactate (15 m*M*). pH was adjusted to 7.4. In similar experiments, the effect of the NADPH oxidase inhibitor diphenylene iodonium (DPI, 10 μM) and the oxidant donor SIN-1 (1 m*M*; 3-morpholino-syndnonimine) was tested. Note the differences in VEGF in response to lactate supplementation.

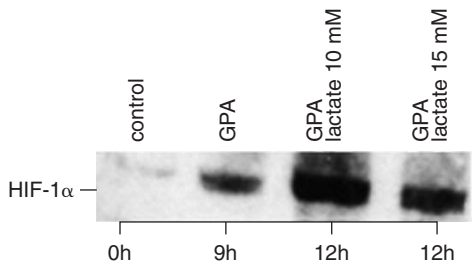

FIG. 8. Lactate-induced stabilization of HIF-1α in oxygenated endothelial cells. Endothelial cells were grown in six-well plates to subconfluent monolayers with a cell density of $\sim 10^6$ cells/well. After overnight serum starvation, cells were treated for 9 h with the prolyl-hydroxylase inhibitor GPA1734 (8,9-dihydroxy-7-methyl-benzo•quinolizinium bromide; kindly provided by Dr. M. Maragoudakis, Patras, Greece), and HIF-1α protein was detected by Western blotting. For immunoblotting, cells were washed with ice-cold PBS and collected by scraping into 1 ml PBS. After centrifugation at 1,000 *g* for 5 min, pellets were homogenized with an equal volume of lysis buffer (20 m*M* Tris-HCl, pH 7.5, containing 150 m*M* NaCl) (Cell Signaling, Beverly, MA) containing protease inhibitors and incubated for 20 min on ice. After sonication for 15 s, samples were subjected to centrifugation at 15,000 *g* for 20 min at 4°C to separate membrane and cytosolic fractions, suspended in 2x SDS sample buffer (Cell Signaling, Beverly, MA) containing 1 m*M* DTT, and boiled for 5 min. Equal quantities of protein were separated by SDS-PAGE electrophoresis under reducing conditions by using a 7.5% Tris-HCl gel (Bio-Rad, Hercules, CA). After transfer onto 0.45-μm nitrocellulose membranes (Millipore, Marlborough, MA), the membranes were blocked with 5% nonfat dry milk and probed with anti HIF-1 (1:1,500; Novus Biologicals, Littleton, CO). GPA 1734 was used to block the proteosomal degradation pathway of HIF-1 α.

chondria, lysosomes, or peroxisomes. It was thus concluded that generation of ·OH in a Fenton reaction at the ER contributes to HIF-1α regulation by either inhibition of prolyl hydroxylase activity or interference with redox-sensitive residues. Thus, under normal physiologic conditions, changes of the redox status within a cell may contribute to an efficient and fast-responding oxygen-sensing system. The current data go further to suggest that lactate, which may be derived from multiple aerobic sources, increases the sensitivity of this system by binding to iron and enhancing ·OH flux, thus preparing for neovascularization under the threat if not the fact of hypoxia (22). In this sense, it is plausible that the degree of hypoxia to which the HIF mechanism responds depends on the amount of lactate that is present in the environment. It appears that considerable, apparently contradictory, experimental evidence may be resolved by measuring or precisely controlling lactate levels in the relevant experimental systems.

HIF-1α levels in cultured and aerated cells increase in response to the addition of as little as 3 m*M* of the α-hydroxy acids, lactate, oxalacetate, or pyruvate (22). The authors proposed that introduction of any of these glycolytic products would significantly amplify glycolysis, inhibit HIF-1α prolyl hydroxylase(s), and therefore extend the life of HIF-1α. The observation that HIF itself promotes glycolytic metabolism was portrayed as a novel feed-forward signaling mechanism. This concept is defended by our findings demonstrating that elevated lactate induces the production of reactive oxygen species. The reactive oxygen species, in turn, induce VEGF by an HIF-independent mechanism as well (38). H_2O_2, a reactive oxygen species, is now known to be required to support wound angiogenesis and healing (31).

Lactate exerts its effects by at least one other mechanism, by reducing ADPribosylation. The consequence includes many cytoplasmic and nuclear processes, few of them well understood (35, 42). This mechanism clearly regulates angiogenesis and collagen deposition *via* oxidant-independent mechanisms. ADPribosylations decrease in response to lactate accumulation because NAD+ (not NADH) is the source of ADPribose. Excessive lactate accumulation reduces the pool of NAD+ by converting it to NADH by the action of LDH. This is significant because NAD+ is the only substrate for synthesis of ADPribose. Hence lactate diminishes ADPribosylations. Addition of NAD+ reverses the effect of lactate on collagen deposition in cell cultures. Furthermore, the LDH-inhibitor oxamate abolishes the effects of lactate of vascularization.

Ghani (14) proposed a role for LDH and lactate in collagen production on noting that oxamate abrogates the effects of lactate in cultured cells. ADPribose (ADPR) monomer binds to and inhibits collagen prolyl hydroxylase, and increased lactate decreases ADPribosylation and enhances collagen hydroxylation and deposition. Similarly, removal of monoADPR activates VEGF in the absence of protein synthesis (44). Results of experiment 2 confirm some of these results *in vivo*.

Current data on CD117-positive cells indicates that lactate, with oxygen, induces wound angiogenesis in two distinctly different ways. Steep oxygen and lactate gradients in rabbit ear chambers allowed Knighton (19) to demonstrate that new capillaries in wounds sprout from existing, functional vessels where pO_2 is high and grow toward high lactate and low O_2 concentrations toward the core of the wound (19). Reversal of the oxygen gradient halted angiogenesis. Beckert and colleagues (5) demonstrated that lactate enhances cell motility. Thus, a continuous, directional advancement exists of new vessels from old. In the current experiments, however, new vessels in the Matrigel appear to develop also by vasculogenesis, from individual endothelial cells that enter the implant before any connection with preexisting vessels. In lactate-supplemented Matrigel, most tubes were well developed before erythrocytes entered them. Whether, however, erythrocytes enter by angiogenesis or from differentiation of hematopoietic stem cells remains unclear.

The significance of these findings is that lactate has many aerobic sources that would seem to contribute to aerobic angiogenesis or vasculogenesis or both. In all likelihood, this concept reaches beyond wound healing. The data suggest that frequent or prolonged and severe episodes of high lactate in blood or arterial tissue may become a mechanism for arteriosclerosis, perhaps retinopathy, and/or other complications of diabetes in which lactate blood levels run high. Such events combined with "lactate-donating" inflammation from other causes may well accelerate an already established momentum of new vessel formation and matrix deposition. It is pertinent to ask whether lactate might explain, at least in part, why subintimal fibrosis is confined to the arterial system where pO_2 is high. Will minimal or intermit-

tent episodes of lactate accumulation add to the explanation of slowly advancing arterial disease by complementing the redox consequences of episodes of hypoxia in sleep apnea?

SUMMARY AND CONCLUSIONS

1. Accumulated lactate, by itself, is able to initiate angiogenesis and connective tissue synthesis, provided that oxygen is present.
2. Sustained hypoxia inhibits neovascularization.
3. VEGF responds to variations in lactate concentration in wounds from <3 m*M* to at least 5 m*M*.
4. Lactate appears to participate in a normal homeostatic mechanism that enhances sensitivity to metabolic need. However, it cannot be ignored that although lactate accumulation is providential as sensor of metabolic need and a source of healing, it can also instigate undesirable consequences, particularly where inflammation is present and pO_2 is high enough to produce pathologic amounts of connective tissue.

The unique feature of lactate that places it in this powerful position is that it has both aerobic and anaerobic sources, and no matter where or what the source, lactate accumulation stimulates angiogenesis and matrix deposition.

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