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Lactate-sensitive response elements in genes involved in hyaluronan catabolism

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Abstract

Tissue anoxia occurs early in wound healing. This is accompanied by production of lactate followed by increased hyaluronan and CD44 expression, suggesting a cause and effect relationship. Fibroblasts increased hyaluronan and CD44 when lactate was added to cultures. Increased deposition of hyaluronan correlates with greater turnover. In current models of hyaluronan catabolism, it is tethered to cell surfaces by CD44 in caveolin-enriched invaginations. It is cleaved to 20-kDa fragments by Hyal-2 on the plasma membrane, endocytosed, and delivered ultimately to lysosomes, and further digested by Hyal-1. Sequence analyses of promoter regions of genes for CD44, caveolin-1, Hyal-1, and -2 revealed multiple AP-1 and ets-1 response elements. To test their relevance, RNA from lactate-treated fibroblasts was analyzed by reverse transcriptase-polymerase chain reaction. Increased transcripts of c-fos, c-jun, c-ets, Hyal-1, -2, CD44, and caveolin-1 mRNAs were observed. We have thus identified lactate-activated genes important in the wound healing responses. Similar responses facilitating tumor progression, the Warburg effect, may share such mechanisms.

Keywords: Hyaluronan; Hyaluronidase; Hyal-1; Hyal-2; Caveolin-1; CD44; Transcription factors; c-fos; c-jun; AP-1; Lactate

Hyaluronan (HA, hyaluronic acid) is a ubiquitous high molecular weight ($>10^7$ Da) glycosaminoglycan component of the extracellular matrix [1,2]. The HA regulates cell–cell adhesion, orientation, differentiation, and promotes proliferation and movement. It participates in several fundamental biological processes including wound healing, repair and regeneration, inflammation, embryological development, and tumor progression (for reviews, see [3–5]). CD44, a multifunctional cell surface glycoprotein, is the predominant receptor for HA [6–8].

In the course of adult wound repair, concurrent with the loss of blood supply, pO_2 levels drop as lactate production and HA levels increase. Extracellular HA levels reach a maximum at 3–4 days [9]. The temporal sequence suggests a cause-and-effect relationship. We documented previously that lactate added to cultured dermal fibroblasts stimulates HA production as well as CD44 expression [10]. This is an important biological mechanism, relevant not only for wound healing and the stress response to injury, but also during embryogenesis and the anaerobic glycolysis associated with neoplasia. The ability of tumor cells to produce lactate, even in the presence of adequate supplies of oxygen, is known as the Warburg effect, named after the investigator who first described this phenomenon over 75 years ago [11–13].

Some of the redox-sensitive regulatory mechanisms that occur at the level of gene transcription may be lactate-responsive elements. Control of CD44 expression also appears to be mediated by lactate-sensitive regulatory mechanisms. Prominent among potential factors are fos, jun, and the ets family of eukaryotic transcription factors [14,15]. Levels of such factors in cultured human dermal fibroblasts and their response to lactate were examined in the present studies.

Turnover rates of HA are normally high [16] and increase even more as levels of HA increase. The catabolic component of HA turnover remains elusive. The hyaluronidases involved, relatively neglected until

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recently [17], are now beginning to be characterized [18,19]. Current models invoke the somatic hyaluronidases Hyal-1 and -2, with the participation of CD44. CD44 tethers high molecular weight HA of the ECM to the cell surface. Hyal-2, a GPI-(glycosylphosphatidylinositol) enzyme anchored to the cell surface cleaves high molecular weight HA to 20 kDa intermediate-sized fragments [20,21]. The HA is endocytosed in membrane regions rich in caveolin, termed lipid rafts [22]. Transcription patterns of the genes for Hyal-1, -2, and for caveolin-1 were examined for their response to lactate in cultured fibroblasts, as well as their promoter regions for lactate-sensitive c-fos, c-jun, and ets-1 response elements.

Materials and methods

Cell culture. Human dermal fibroblasts, HS27, obtained from the ATCC, were grown routinely in DME with 10% FCS with 5% CO_2 on four-chamber plastic plates. Cells were washed with Hank's basic salt solution and media were replaced with 0.5 ml medium plus the indicated additions of lactate.

Reverse transcriptase-polymerase chain reaction. The RNA obtained from 2 to 4×10^6 cells using RNA Track (Biotecx, East Houston, TX) was used as a template for the first strand of cDNA in a reaction mixture containing 1000 U Maloney murine leukemia virus reverse transcriptase (Life Technologies, Rockville, MD), 50 μM random hexamer primer, 50 mM Tris–HCl (pH 8.3), 75 mM KCl, 10 mM dithiothreitol, 3 mM $MgCl_2$, and 0.5 mM of each deoxynucleotide in 60 μl. The reaction was carried out at 37 °C for 90 min and then diluted 1:3 or 1:7 in water pretreated with DEPC. Oligonucleotide primers were diluted to 40 μM in water. For RT-PCR, a modified hot start method was used. A quantity of 3–5 μl of cDNA was added to 30 μl of a master mix containing 0.8 μmol of each primer, 17 mM Tris–HCl (pH 8.3), and 80 mM KCl. Water was added to the cDNA reaction mixture to a final volume of 40 μl. All reactions were overlaid with light mineral oil and heated to 99 °C. After 10 min, the reactions were cooled to 94 °C, and a 10 μl volume containing 7 mM $MgCl_2$, 1 mM each of dATP, dTTP, dGTP, and dCTP and 1.2 U Taq polymerase (Perkin–Elmer–Cetus, San Jose, CA) was added directly through the oil overlay. Cycle parameters were 95 °C for 60 s; 55–57 °C for 30 s, 72 °for 30 s, with a 15-min final extension at 75 °C. Reactions were cycled 30–32 times using an automated programmed thermocycler (Perkin–Elmer). RT-PCR products were separated on 2% agarose and the ethidium bromide gels run at 200 V for 45 min.

The oligonucleotide primer sequences used in the RT-PCR to amplify the human fos gene (GenBank Accession No. #K00650M16287) were: F (forward primer) 5′-CACGGACCTGG CCGTCTCCA-3′ and for the R (reverse primer), 5′-CTTGGAGTG TATCAGTCAGC-3′ amplifying a product of 356 bp. For the human ets-1 gene (GenBank Accession No. #X63279), F (forward primer), 5′-ACGCTTTTG GGCCGC-3′ and R (reverse primer), 5′-CTCGGGTC CCAGCC-3′ amplifying a product of 407 bp. For the human jun gene (GenBank Accession No. #J04111), F (forward primer), 5′-CAAA GATGGA AACGACCT-3′ and R (reverse primer), 5′-TCTGTCACGTTCTT GGGGC-3′, amplifying a product of 307 bp.

For human CD44s, the standard form, with exons 5 and 15 in tandem, F5 (forward CD44 exon 5), 5′-GATGATGACGTGAGC GCTC-3′ and R15 (reverse CD44 exon 15), 5′-ATTCAGAGATCCA TGAGTGGTA-3′, producing a transcript of CD44 of 201 bp.

For the human Hyal-1 gene, HYAL1 (GenBank Accession No. #U96078), F (forward primer), 5′-ACCGCATAGTCAAACAGG-3′ and R (reverse primer), 5′-GCACATAATCATGATTG-3′, amplifying a product of 247 bp.

For the human Hyal-2 gene, HYAL2 (GenBank Accession No. #NM003773), F (forward primer), 5′-ACCGCATAGTCAAACAG G-3′ and R (reverse primer), 5′-GCACATAATCATGATTG-3′, amplifying a product of 147 bp.

For the human caveolin-1 gene (GenBank Accession No. #NM001753), F (forward primer), 5′-CGACCTGGTCAACCGCG A-3′ and R (reverse primer), 5′-AAGTAAATGCCCCAGATGA-3′, amplifying a product of 210 bp.

For controls, and to demonstrate equal loading, both for the β-actin control and the RANTES receptor genes were used.

The β-actin control primer sequences were (GenBank Accession No. #NM00101), F (forward) 5′-GCTCTCTTCCAGCCTTCC-3′, and R (reverse) 5′-AGAGCCACCAACCCACACAGAG-3′ amplifying a product of 252 bp.

The human RANTES receptor gene primers were (GenBank Accession No. #L10918), F (forward primer), 5′-ACAGAGGACTAT GACCA-3′, and R (reverse primer), 5′-GAGGATCTTACACATG GCA-3′, amplifying a product of 310 bp.

Primer pairs were designed to span introns to avoid amplification of potential contaminating genomic DNA.

Results

The CD44 family of adhesion proteins are products of a single gene, all generated by variant exons spliced into a single site [23]. The transcription of the gene for CD44s, the standard form without variant exons, was enhanced when human dermal fibroblasts were cultured for 20 h in the presence of increasing levels of lactate, in a dose-dependent manner. Subplateau RT-PCR was used to analyze mRNA transcripts. Fig. 1A indicates

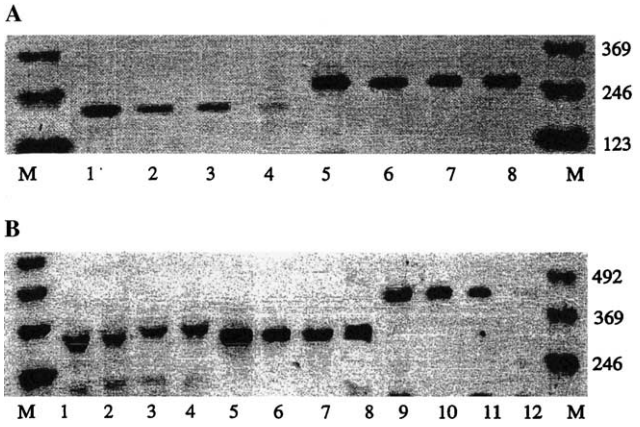

Fig. 1. Effect of increasing concentrations of lactate on steady-state levels for CD44s, c-fos, c-jun, ets-1, and β-actin in human dermal fibroblast cultures, as determined by RT-PCR. (A) cDNA for CD44s amplified with primers to produce a 201-bp product (lanes 1–4) at 20, 10, 5, and 0 mM lactate. Lanes 5–8, transcripts of the 252-bp β-actin control. (B) Expression of mRNAs for c-fos (lanes 1–4, with a 356-bp product), c-jun (lanes 5–8, with a 307-bp product), and c-ets-1 (lanes 9–12, with a 407-bp product) at 20, 10, 5, and 0 mM lactate, respectively. The same cDNA extracts were used and the β-actin controls are relevant for all experiments. The RT-PCR run was 32 cycles with an annealing temperature of 57 °C.

that CD44s was up-regulated, confirming earlier results [10]. Lanes 1–4 represent mRNA expression in 20, 10, 5, and 0 mM lactate, concentrations that are in the physiological range. The β-actin control showed no change in transcription in the presence of lactate at these concentrations, lanes 5–8.

Sequence analysis of the CD44 promoter region revealed presumptive response elements (TGAC/GTCA) for the transcription factor AP-1, a heterodimer of c-fos and c-jun [14]. We also observed that the promoter region had two hitherto unrecognized ets-1 response elements (GAGGA) [24]. Response elements in the promoter regions of the genes for c-jun, c-ets-1, HYAL1, HYAL2, and caveolin-1 were also found. The number of response elements for these genes are summarized in Table 1. The positions of the transactivating binding sites in these genes are given in Table 2.

Transcription of genes for transcription factors c-fos (Fig. 1B, lanes 1–4), c-jun (lanes 5–8), and ets-1 (lanes 9–12) were enhanced in the presence of lactate, at 20, 10, 5, and 0 mM, respectively.

As shown earlier, levels of HA increased as fibroblasts were exposed to increasing levels of lactate. Turnover rates of HA increase as levels of HA increase. The catabolic scheme for HA invokes the participation of somatic hyaluronidases Hyal-1 and -2, as well as CD44 and caveolin-rich membrane microdomains, termed lipid rafts [22]. As shown, transcripts for Hyal-2 increased as lactate increased, from 0, 5, 10 to 20 mM (Fig. 2C, lanes 1–4). Transcripts for the RANTES receptor did not increase (Fig. 2A, lanes 1–4), while c-fos, used as a positive control, did increase (Fig. 2B, lanes 1–4).

Transcription of the caveolin-1 gene was also examined. Little change was observed as lactate was increased

Table 1
Promoter region response elements

Gene	ets-1 (GAGGA)	AP-1 (TGAC/GTCA)
CD44	2	1
c-jun	0	1
c-ets-1	1	1
HYAL1	1	4
HYAL2	1	2
Caveolin-1	1	3

Table 2
Transactivating binding sites

Gene	ets-1 positions	AP-1 positions
CD44	110–114	889–892; 968–971
c-jun	None	381–384
c-ets-1	482–485	1206–1209
HYAL-1	84–88	176–179; 268–271; 302–305; 534–537
HYAL-2	6–10	16–19; 107–110
Caveolin-1	325–329	224–227; 308–311; 761–764

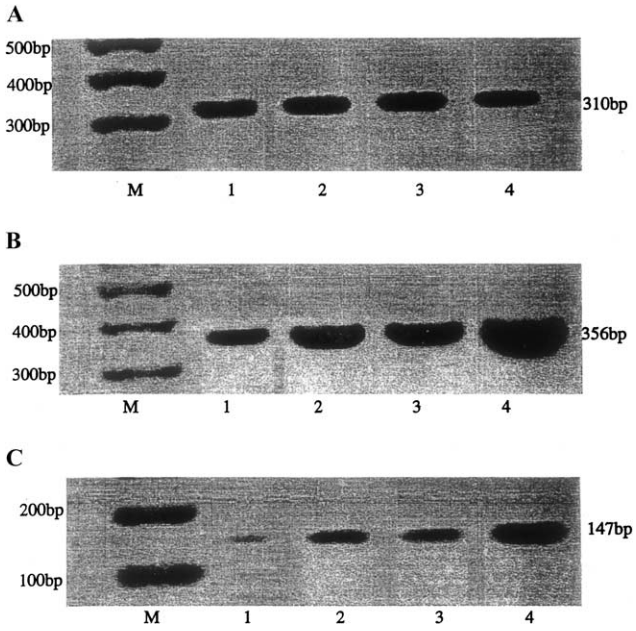

Fig. 2. Effect of lactate on expression of mRNA for Hyal-2. (A) cDNA for RANTES amplified with primers to produce a product of 310 bp, using HS27 fibroblasts cultured in the presence of 0, 5, 10, and 20 mM lactate, in lanes 1–4, respectively. (B) For positive controls, cDNA amplified with c-fos primers to produce a product of 356 bp from fibroblasts cultured with increasing lactate (lanes 1–4) as in (A). (C) cDNA amplified with Hyal-2 primers to produce a product of 147 bp, from fibroblasts cultured with increasing lactate (lanes 1–4). Note significant up-regulation of Hyal-1 transcript in 20 mM lactate in lane 4.

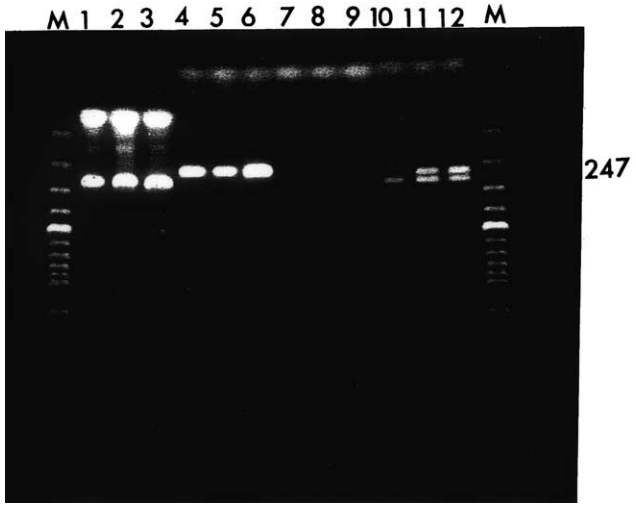

Fig. 3. Effect of lactate on expression of mRNA for Hyal-1 and caveolin-1. cDNA for the β-actin control amplified with primers to produce a 252-bp product using HS27 fibroblasts cultured in the presence of 0, 10, and 20 mM lactate, shown in lanes 1–3, respectively. cDNA amplified with caveolin-1 primers from cells under the same condition are shown in lanes 4–6. Lanes 7–9 demonstrate the absence of cDNA, to control for possible genomic DNA contamination. In lanes 10–12, cDNA amplified with primers for Hyal-1 to produce two products of 220 and 247 bp, again using HS27 fibroblasts under conditions of 0, 10, and 20 mM lactate, respectively. M represents the lane for DNA molecular weight markers in each of the figures.

from 0 to 10 mM. However, a major increased in transcription was seen at 20 mM lactate (lanes 4–6, respectively). Transcription of the β-actin gene remained constant as lactate increased (lanes 1–3). Transcripts for Hyal-1 also increased with increasing lactate. As shown in Fig. 3, Hyal-1 transcripts were up-regulated (lanes 10–12) as lactate added to the fibroblast cultures was increased from 0 to 10 and 20 mM, respectively. Two bands are apparent in the gel, at approximately 220 and 247 bp reflecting possible splice variants [25,26]. Both classes of transcripts were up-regulated with increasing lactate. Lanes 7–9, in which no bands can be seen, are controls without RT addition, to test for possible DNA contamination.

Discussion

The expression of most hypoxia-inducible genes is mediated by the transcription factor HIF-1, involving a heme-protein-dependent step [15]. Although oxygen sensing plays a substantial role, it is not the only mechanism [27]. A potential alternative signaling pathway involves changes in the redox state of cells [28], supported by the observation that GLUT-1 mRNA [29] as well as c-fos and c-jun [30] can be up-regulated by either lactate or by hypoxia. Lactate induces increased NAD(P)H/NAD(P) ratios [31], in a manner similar to exposure to hypoxia [32,33].

As we reported earlier [10], tumor cells generate lactate in the presence of adequate oxygen supplies, known as the Warburg effect. Hyaluronan facilitates tumor cell spread [34–36]. Generation of lactate is a mechanism by which cancer cells induce peritumor fibroblasts to produce HA, a mechanism by which tumor cells can commandeer the host to participate in its own destruction. Evidence is provided in the current studies that the increased turnover of HA, associated with elevated levels of HA production, is linked to lactate-sensitive response elements in genes for CD44, caveolin-1, Hyal-1, and Hyal-2 (Tables 1 and 2). We also document here that transcription of these genes was stimulated after exposure to lactate.

The AP-1 binding factor, the fos-jun heterodimer, binds a specific DNA consensus sequence, dependent on the redox state of a specific cysteine residue within the highly conserved lys–cys–arg DNA-binding domain [37]. Reduction of this cysteine residue is necessary for DNA-binding, while its oxidation blocks binding [30,38]. Of additional interest is a putative mechanism whereby lactate interacts with the cysteine adduct, perhaps through its α-hydroxy structure. We have recently surveyed a range of α-hydroxy acids and find that many of these enhance HA production in cultured fibroblasts (B. Neudecker and R. Stern, submitted).

We observed previously that lactate enhances mRNA and protein levels of CD44 [10]. A presumptive AP-1 binding site exists in the promoter region at nucleotides −108 to −114 of the CD44 gene [39], perhaps providing a mechanism for its up-regulation, by means of the enhanced gene expression of c-fos and c-jun in response to lactate (see Table 1 and Fig. 1). Our results confirm those of Lamb et al. [40], who report that increased expression of CD44 in fos- and EGF-transformed cells is dependent upon AP-1, as well as those of Hofmann et al. [41] who document that the promoter in the CD44 gene is AP-1 responsive.

The ets family of transcription factors consisting of approximately 30 members, contain the highly conserved GAGGA DNA-binding motif [42]. They share a DNA-binding domain that interacts with a core nucleotide sequence GGA. The human ets-1 gene promoter region has an AP-1 binding site and one ets-1 protein-binding site, indicating an auto regulatory mechanism for ets-1 gene expression [43]. In the present study, we demonstrated that lactate enhances ets-1 mRNA levels. The CD44 promoter region has two ets-1 binding sites, suggesting that ets-1 contributes to the enhanced transcription of the CD44 gene in response to lactate.

Increased HA turnover involves the two somatic hyaluronidases, Hyal-1 and -2. As demonstrated here, transcription of the gene for each of these enzymes is stimulated by lactate. Each of these genes has a single ets-1 response element, as well as four and two AP-1 sites, respectively. We speculate that the stromal fibroblasts that surround malignant tumors as well as those in healing wounds secrete higher levels of hyaluronidase in response to increasing levels of lactate. Thus, the lactate produced by the tumor cells is pivotal in recruiting peritumor fibroblasts to enhance the metastatic potential of the malignancy. High molecular weight is a space-filling, anti-angiogenic, and anti-inflammatory molecule [44]. The hyaluronidases responding to the increased lactate degrade the HA to smaller fragments, which in turn are highly angiogenic and very inflammatory [45].

Many parallels exist between wound healing and tumor progression [13]. We suggest that this unique lactate-responsive pathway for HA catabolism is common to both wound healing and tumor progression, and constitutes a portion of the parallels that exists between these two processes.

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