THE ABNORMAL HYALURONAN CATABOLISM IS INVOLVED IN THE DEVELOPMENT OF CHRONIC ULCERS IN DIABETES MELLITUS

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ABSTRACT

It is generally accepted that hyaluronan (HA) is implicated in wound healing regulation, but its role in the formation of chronic diabetic ulcers is poorly understood. Earlier we demonstrated that fibroblasts of non-injured skin of diabetic (Type-2) patients with ulcers, unlike cells from patients without ulcers, accumulate the excessive amount of high-molecular-weight HA in their extracellular matrix and express high level of CD44. L-lactate, the obligatory component of wound healing process and known modulator of HA metabolism, stimulated CD44 expression and HA accumulation in fibroblasts of both diabetic groups, and the latter was evidently more pronounced in cells of patients with ulcers. The catabolism of HA and its regulation by lactate was not studied in diabetic fibroblasts.

In this work we aimed to compare the expression of hyaluronan synthases (HAS1,2,3) and hyaluronidases (HYAL1,2) in fibroblasts of diabetic patients without and with ulcers and to study the effect of exogenous lactate on the expression of abovementioned enzymes. All fibroblast lines (3 control, 3 diabetic without ulcers and 3 diabetic with ulcers) demonstrated equal level of HAS1,2,3 mRNAs expression. L-lactate treatment (15 μmole/ml, 24 h) up-regulated the expression of HAS2 and 3 and the level of stimulation was the same in all groups of fibroblasts (~1.4-1.5-fold). HAS1 was not altered by L-lactate. Thus, the excessive accumulation of HA by diabetic fibroblasts with ulcers in basal conditions and under lactate stimulation is hardly connected to the increase of HA synthesis. In contrast, diabetic groups of fibroblasts were not the same by the expression of HYAL2, the leading enzyme in HA catabolism. HYAL2 expression in fibroblasts of diabetic patients with ulcers was inhibited (~1.4-fold) both at the level of mRNA and protein comparatively to cells of patients without ulcers and to control ones. Moreover, L-lactate up-regulated the expression of HYAL1, but did not influence HYAL2. As a result, the functional capacity of fibroblasts of diabetic patients with ulcers to degrade the endogenous high-molecular-weight HA was inhibited.

We conclude, that HA catabolism is impaired in fibroblasts of diabetic patients with chronic ulcers, and the deficiency of HA short fragments may be the cause of ulcers development.

Key words: hyaluronan, diabetes mellitus, chronic ulcers

INTRODUCTION

The impaired formation of extracellular matrix (ECM) is involved in the development of chronic ulcers in diabetes mellitus. The properties of ECM are determined mainly by fibroblasts, which, in particular, produce hyaluronan (HA), the leading component of ECM. However, the information about the role of HA in wound healing in diabetes is limited and contradictory [Chen, Abatangelo, 1991]. Earlier we found that fibroblasts of diabetic (Type-2) patients with ulcers accumulate excessive amount of high-molecular-weight (HMW) HA in ECM and express more CD44 (the principal HA receptor) [Yevdokimova, Podpryatov, 2005]. Diabetic fibroblasts produce high level of lactate, and this phenomenon is considered to be involved in the delayed wound healing [Yevdokimova, Podpryatov, 2005; Hegenberger, Hanson, Heilborn, 1999]. Exogenously added L-lactate is known to increase the accumulation of HA, CD44 expression and the expression of mRNAs of hyaluronidase-1 (HYAL1) and -2 (HYAL2) by control fibroblasts [Formby, Stern, 2003]. We demonstrated [Yevdokimova, Podpryatov, 2005] that L-lactate stimulates the accumulation of HA and CD44 expression in diabetic fibroblasts also, but these effects were evidently more pronounced in cells of patients with ulcers. HA catabolism and the expression of HAS and HYAL were not studied for diabetic fibroblasts at all.

Therefore, the aim of current work was to investigate and to compare the expression of HAS1,2,3 and HYAL1,2 in fibroblasts of diabetic (Type-2) patients without and with ulcers and to study the effect of exogenous L-lactate on the expression of enzymes.

MATERIALS AND METHODS

Primary cultures of dermal fibroblasts were established by the explant method from the biopsy material of forearm of three type-2 diabetic patients without ulcers, three patients with ulcers on the upper legs and three nondiabetic volunteers. Metabolic labeling of HA with [^{3}H] glucosamine (6,8 Cimmol sp. act., Amersham Pharmacia Biotech) and the investigation of HA degradation was done as described [Yevdokimova, 2006]. For the determination of the expression of HAS and HYAL mRNAs we used semi-quantitative RT-PCR method. Total RNA was extracted with RNAzolB-kit (AMS Biotechnology). RT-PCR kits were from Invitrogen Ltd and primers – from Integrated DNA Techn. Inc. 2 μg of DNA-free total RNA was converted to cDNA by SuperScript TM II Rnase H-reverse transcriptase with random primers in a 20 μl reaction volume. The reverse transcription reaction

(0.5 μl) was subjected to PCR amplification, using 2.5 U of Taq DNA polimerase in 100 μl reaction volume with 0.5 μM of each dNTP, 0.5 μM of each specific primer, and 1.5 mM $MgCl_2$. The sequences of primers were: for HAS1, HAS2 and HAS3, as in [Yevdokimova, 2006]; for HYAL1, HYAL2 and beta-actin, as in [Jenkins, Williams, Steadman, 2004]. We co-amplified the house-keeping gene of beta-actin to allow semi-quantificative comparison of PCR products. 0.5 ml of

amplified RT cDNA was nonsaturating for the PCR product of all genes. 3 min of denaturation at 94^0C was followed by 32 PCR cycles (60 s at 94^0C, 60 s at 62^0C and 60 s at 72^0C) and final extension (10 min, 72^0C). 10µl of PCR reaction product was electrophoresed through a 1.2% agarose with 0.5 µg/ml ethidium bromide. The expression of HYAL1 and 2 proteins were studied by Western blotting. After cell lysis and dilution with reducing loading buffer samples were resolved on 10% SDS/PAGE, transferred onto nitrocellulose membrane and incubated (4^0C, overnight) with polyclonal primary antibodies to HYAL1 and 2 (1:300 for both, Santa Cruz Biotech. Inc). The binding of secondary antibodies was revealed by ECL+Plus. The results were normalized against the intensity of beta-actin band.

Results were compared using unpaired t-test. P values of < 0.05 (or less) were regarded as significant.

RESULTS

The accumulation of matrix [^{3}H]HA in diabetes with ulcers in comparison to control was increased that was caused only by the augmentation of HMW fraction, while cells of patients without ulcers did not differ from the control, confirming our previous results with other cell lines [Yevdokimova, Podpryatov, 2005].

It is seen (Table I) that all fibroblasts express three types of HAS, and the expression of each enzyme is the same for control and diabetic groups.

Table 1 The expression of HAS mRNA by control fibroblasts and cells of diabetic patients without and with ulcers. Effect of exogenous L-lactate.

HAS	control	control+ lactate	diabetes	diabetes+ lactate	diabetes+ulcers	diabetes+ulcers +lactate
1	0.59±0.07 (n=9)	0.64±0.09 (n=6)	0.71±0.10 (n=9)	0.62±0.08 (n=6)	0.65±0.08 (n=9)	0.57±0.09 (n=6)
2	1.29±0.15 (n=9)	1.94±0.21* (n=6)	1.23±0.15 (n=9)	1.75±0.19* (n=6)	1.31±0.14 (n=9)	1.88±0.20* (n=6)
3	1.04±0.12 (n=9)	1.46±0.16* (n=6)	0.99±0.11 (n=9)	1.39±0.15* (n=6)	1.11±0.13 (n=9)	1.63±0.18* (n=6)

Cells were incubated with or without 15 µmole/ml L-lactate for 24 h. Results are expressed as mean ± SE of the fluorescence ratios of each product normalized to those of beta-actin.* - $P<0.05$, as compared to basal conditions (the absence of exogenous lactate).

In contrast, the expression of HYAL2 mRNA, unlike HYAL1, was significantly suppressed in fibroblasts from diabetic patients with ulcers. (Table II). Western blotting analysis demonstrated also the significant suppression of the level of HYAL2 protein by fibroblasts from diabetic patients with ulcers (Fig.1).

Table 2 The expression of HYAL mRNA by control fibroblasts and cells of diabetic patients without and with ulcers. Effect of exogenous L-lactate.

HYAL	control	control+ lactate	diabetes	diabetes+ lactate	diabetes+ulcers	diabetes+ulcers+ lactate
1	0.73±0.09 (n=9)	0.96±0.11^{+} (n=6)	0.69±0.08 (n=9)	1.07±0.12^{+} (n=6)	0.74±0.09 (n=9)	0.97±0.14^{+} (n=6)
2	0.96±0.12 (n=9)	1.24±0.14^{+} (n=6)	0.99±0.12 (n=9)	1.29±0.15^{+} (n=6)	0.71±0.09*** (n=9)	0.66±0.11 (n=6)

The incubation and data expression were as in Table I. *** - P<0.01, as compared to control; $^{+}$ - p<0.05, as compared to basal conditions.

Exogenous L-lactate stimulated the expression of HAS2 and 3 in fibroblasts of all groups (Table I). L-lactate stimulated also the expression of HYAL by control cells and fibroblasts of diabetic patients without ulcers (Table II). Fibroblasts of diabetic patients with ulcers were insensitive to lactate in terms of HYAL2 mRNA expression, in contrast to HYAL1 mRNA expression.

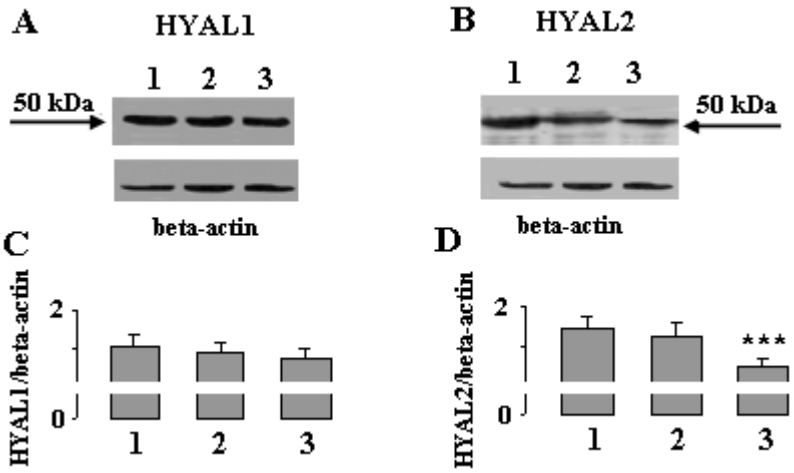

Figure 1 The expression of HYAL1 and HYAL2 proteins in control fibroblastst and cells of diabetic patients without and with ulcers.

Panels A and B – the typical example of Western blotting analysis. Panels C and D – the ratio of HYAL band absorbance to beta-actin band absorbance. 1 – control fibroblasts, 2 – fibroblasts of diabetic patients without ulcers, 3 - fibroblasts of diabetic patients with ulcers. Data are expressed as mean ± SE of three experiments with all cell lines studied, *** - P<0.01.

L-lactate did not influence also the expression of HYAL2 protein: the ratio to beta-actin band absorbance was 1.31±0.16 (n=6, p>0.05, comparatively to basal conditions, see Fig.1).

The degradation of exogenous HMW HA by fibroblasts of diabetic patients with ulcers (but not without ulcers and control cells) was decreased under L-lactate treatment (Fig.2).

DISCUSSION

HA is a linear polymer of glucouronic acid and N-acetylglucosamine disaccharide, which is synthesized by HAS (1,2 and 3) at the inner side of plasma membrane. HAS1 and 2 produce chains of a similar large size (>2000 kDa), whereas HAS3 synthesizes shorter chains (~200 kDa). HA is cleaved into fragments of low and intermediate molecular weight by HYAL1 and 2. HA is tethered to cell surfaces by CD44, cleaved to 20 kDa fragments by HYAL2, delivered to lysosomes and further digested by HYAL1 [Stern, Asari, Sugahara, 2006]. Unlike the native HMW polymer, HA fragments have immunostimulatory, angiogenic and antiapoptotic effects [Stern, Asari, Sugahara, 2006], and were demonstrated to promote fibroblast proliferation [Huang, Gu, Burd, 2009]. All abovementioned properties of HA fragments are quite important for adequate wound healing [Chen, Abatangelo, 1991].

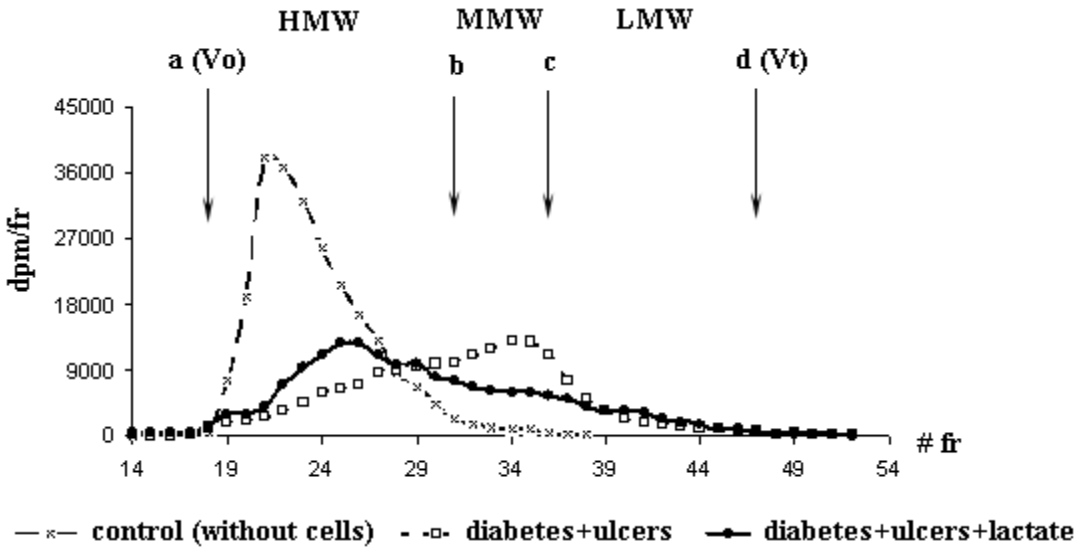

Figure 2 Effect of L-lactate on the degradation of HMW [^{3}H]HA by fibroblasts of diabetic patients with ulcers.

HMW [^{3}H]HA (~250000 dpm) was incubated with fibroblasts of diabetic patients with ulcers with or without 15 μmole/ml L-lactate for 72 h. [^{3}H]HA was isolated from the conditioned medium and chromatographed on a 1x30 cm Sephacryl S-1000 column in 0,1 M Na-acetate buffer, 0.025% CHAPS, pH 6.5. Fraction volume was 0.5 ml. Arrows mark the position of the compounds used for column calibration: a (Healon GV, 7000 kDa) at fr 18, b (blue dextran, 2000 kDa) at fr 31, c (decorin, 100 kDa) at fr 36, d ([^{3}H]glucosamine hydrochloride, 215 Da. Three arbitrary size brackets of HA are indicated, high molecular weight (HMW) - >2000 kDa; middle (MMW) – 100-2000 kDa; and low (LMW) - <100 kDa.

In this work we demonstrated that the expression of mRNAs of all HAS were the same in control and diabetic cells, therefore the increased production of HA by diabetic cells of both groups and

elevated accumulation HMW [^{3}H]HA in ECM of fibroblasts of diabetic patients with ulcers were not determined by an increase of HA synthesis. It is evident that accumulation of HA may be the consequence not only of the elevated synthesis, but of the suppressed degradation. We observed that the expression of the leading hyaluronidase (HYAL2) at the level of mRNA and protein was decreased (~1.5-fold) in fibroblasts of diabetic patients with ulcers comparatively to control cells and fibroblasts of diabetic patients without ulcers. Naturally, the functional capacity of fibroblasts of diabetic patients with ulcers to degrade exogenously added HA was suppressed also.

L-lactate treatment stimulated the expression of mRNAs of HAS2 and 3 in all studied groups of fibroblasts, being in line with the results of other works [Formby, Stern, 2003; Rudrabahata, Mahaffey, Mummert, 2006]. However, lactate did not influence the expression of HYAL2 at the level of both mRNA and protein in fibroblasts of diabetic patients with ulcers contrary to its effect on diabetic fibroblasts of patients without ulcers and control cells. The expression of HYAL1 mRNA was up-regulated by lactate treatment for all cell lines. Since the degradation of HA begins from the action of HYAL2, the effect of HYAL1 may be partly blocked in case of fibroblasts of diabetic patients with ulcers under lactate treatment. Exogenous lactate inhibits the functional capacity of fibroblasts of diabetic patients with ulcers to degrade exogenous HMW HA, and this phenomenon confirms our suggestion.

CONCLUSION

We demonstrated the impaired regulation of HA catabolism in fibroblasts of diabetic patients with ulcers. Therefore, possibly, that in such patients, the tissue injury, which stimulates lactate production, simultaneously promotes the accumulation of HMW HA and the shortage of HA with smaller molecular mass. The latter may be involved in the transformation of acute wounds into chronic ulcers.

Acknowledgments

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