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Redox status of cultured fibroblasts. Possible relations with specific catabolic rates of proteoglycans[1])

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With one figure

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Abbreviations: CS = chondroitin sulfate; DS = dermatan sulfate; GAG = glycosaminoglycans; GSH = glutathion reduced; S.A. = specific activity (^{35}S-sultate); PG = proteoglycan, a core protein linked with GAG chains

Summary

In cultured embyonic rat fibroblast the cytoplasmic NAD/NADH ratio was determined from the lactate/pyruvate ratio under acidic, hypoxic and lactic acid-rich conditions.

Results and conclusions:

1. The NAD/NADH ratio is reduced when the lactate concentration increases at *p*H 7.4 with and without hypoxia.
2. At *p*H 6.6 this ratio is not reduced by lactate in normoxia: Conditions of aerobic glycolysis did not increase NADH.
3. The NAD/NADH ratio was strongly lowered at *p*H 6.6 by lactate plus hypoxia. At low cell density this condition of hypoxic glycolysis is correlated with the increase of the specific activities of CS- and DS-proteoglycans (DS $\gg$ CS). But only the CS concentration was increased.
4. Conditions of aerobic glycolysis at low cell density caused a moderate increase of both the specific activity and concentration of DS.
5. The different regulation of the turnover of CS- and DS-proteoglycans is suggested to be based on their different synthetic capacities (CS > DS) on the one side and on the effect of specifically acting proteoglycanases on the other side.
6. Maximal degradation of proteoglycans seems to be stimulated by NADH-activated proteases, supposedly thiol proteases. Lower degradation seems to be effectuated under non-reductive acidic conditions. Both these types of degradation seem stronger to affect DS-proteoglycan. Some neutral proteases seem stronger to affect CS-proteoglycan.
7. Improved oxygen supply might reduce the CS concentration in proliferating tissue, such as in chronic inflammation, and thus reduce this process.

Introduction

Glycosaminoglycans and proteoglycans play important roles in the growing and resting states of normal, inflamed and newly formed tissues (e.g. Nemeth-Csoka 1972; Baig et al.

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1978; CHIARUGI et al. 1978; SAMPAIO and DIETRICH 1981; DIETRICH et al. 1982; IOZZO et al. 1982; FIBBI et al. 1983).

That is why we are dealing with the levels and turnover rates of GAG in rheumatoid tissue and in fibroblasts cultured under non-physiological conditions. In these investigations a different behaviour of CS and DS has been observed (KITTLICK 1977b; KITTLICK et al. 1980; KITTLICK and NEUPERT 1982a, b). Some of these results prompted us to determine the intracellular concentration of lactate and pyruvate and in this way the cytoplasmic NAD/NADH ratio in appropriate fibroblast cultures.

The data obtained suggest that aerobic and anaerobic glycolysis are highly important conditions for the control of the CS/DS ratio in normal and inflamed tissues. It is proposed that the NADH level controls PG-degrading enzymes. Specific PG degradation followed by specific PG neosynthesis results in an alteration of the GAG- and PG-pattern.

Material and Methods

Hepes, p.a., and L-lactate sodium were obtained from Serva Heidelberg/FRG, Eagle basal medium from the Institut für Immunpräparate, Berlin-Weißensee/GDR; LDH, NAD and NADH from Arzneimittelwerk Dresden/GDR. Calf serum was provided by Dr. G. NEUPERT.

Embryonic rat fibroblasts (1st to 6th subculture) in Eagle basal medium plus 10% calf serum and buffered with 0.03 M Hepes to *p*H 7.4 or 6.6, were grown as monolayers in demeter flasks (50 cm^2) to the cell density desired. The medium was changed every two days (NEUPERT et al. 1972). When required 100 mg% L-lactate were added to the medium before adjusting the *p*H-value. Hypoxia (1.4 to 2.6 vol% O_2) was attained by applying to the flasks a defined argon stream under defined conditions. In companion flasks without cells the O_2 concentration was controlled using an oxygen analyzer M65F (VEB Metra, Radebeul/GDR) (KITTLICK 1977a).

The cells were cultured in the experimental media at least for 2 d. Then they were scraped off with a rubber policeman in 2.5 ml of the same (conditioned) medium and throughly dispersed with a Pasteur pipette. The cells were counted in blood cell counting chambers (Fuchs-Rosenthal) after dilution with phosphate-buffered saline (PBS) plus 0.02% versene. The remaining bulk of cells was isolated by rapid filtration on small glass fiber papers and rapid rinsing (4 times) under suction. For hypoxic conditions argon was provided up to this point. These filter papers were transferred into 1 ml of 6% $HClO_4$ and homogenized manually at 4 °C. After 1 h the $HClO_4$ extract was removed and neutralized with 2M K_2CO_3 solution. Parts of the supernatant (0.1—0.3 ml) were taken to determine lactate and pyruvate concentrations. For determination of lactate NAD, hydrazine-glycine buffer (*p*H 9.5) and versene (1.0 ml final vol., BERGMEYER 1970, p. 1425), for determination of pyruvate NADH, triethanolamine buffer (*p*H 7.5) and versene (0.8 ml final vol., BERGMEYER 1962, p. 253) were used. The reactions were started with LDH and recorded at 334 mm with the universal spectrophotometer VSU 2-P (VEB Carl Zeiss JENA).

Results

In the cytosol of liver cells 3 separate pools of NADH have been demonstrated (cp. SIES 1982). We have determined the lactate/pyruvate-linked pool using LDH and thereafter the NAD/NADH ratio with the formula $\frac{(L)\cdot(NAD)}{(P)\cdot(NADH)} = \frac{1}{1.11\times10^{-4}}$ according to KREBS and VEECH (1969).

The results obtained with embryonic rat fibroblasts under different *in vitro* conditions are shown in table 1. As expected, both lactate application and hypoxia enhance NADH at the expense of NAD. However, there is evidence that a decrease of the *p*H value of the culture medium from 7.4 to 6.6 at normoxia does overcome the lactate effect and leads to a NAD/NADH ratio of 1629. This means that in conditions of aerobic glycolysis (21% O_2. low *p*H, high lactate concentration) an elevated NADH level does not exist. This result is in agreement with the increased O_2 consumption of cultured fibroblasts at *p*H 6.6 (150%: KITTLICK and NEUPERT 1973) and the increased lactate oxidation below *p*H 7.2 (PAUL 1965).

In fig. 1, which also includes earlier results, it is demonstrated that under the conditions of both hypoxic and normoxic glycolysis the turnover rate (S.A.) of DS-PG clearly exceeds that of CS-PG. This difference is particularly high when the NAD/NADH ratio is low (423 in conditions of hypoxic glycolysis).

Table 1. The NAD/NADH ratios in embryonic rat fibroblasts cultured under physiological and non-physiological conditions. The ratios were derived from the lactate and pyruvate concentrations within 10^6 cells after KREBS and VEECH (1969); see "*Results*"

Culture medium			Proliferating cultures				Dense cultures			
*p*H	lactate[1])	$\%O_2$	lactate[2])	pyruvate[2])	$\frac{\text{pyruvate}}{\text{lactate}}$	$\frac{\text{NAD}}{\text{NADH}}$	lactate	pyruvate	$\frac{\text{pyruvate}}{\text{lactate}}$	$\frac{\text{NAD}}{\text{NADH}}$
7.4	—	21	0.0190 ± 0.0057 (5)	0.00257 ± 0.00086 (4)	0.1345	1212	0.0102 ± 0.0045 (5)	0.0013 ± 0.0006 (5)	0.1275	1148
7.4	—	2					0.0270 ± 0.0017 (2)	0.00245 ± 0.00040 (2)	0.0907	817
7.4	100	21	0.0840 ± 0.0077 (2)	0.00595 ± 0.00120 (2)	0.0708	638	0.0375 ± 0.0144 (2)	0,0037 ± 0.0011 (2)	0.099	892
7.4	100	2	0.0384 ± 0.0125 (5)	0.0029 ± 0.0014 (5)	0.0755	680	0.0193 ± 0.0041 (2)	0.00115 ± 0.00030 (3)	0.0596	536
6.6	100	21	0.0330 ± 0.0140 (3)	0.0060 ± 0.0027 (3)	0.180	**1629**				
6.6	100	2	0.0560 ± 0.0076 (3)	0.00263 ± 0.0007 (4)	0.047	**423**	0.0254 ± 0.0030 (3)	0.00168 ± 0.00030 (3)	0.0661	596

1) mg% L-lactate
2) μMol per 10^6 cells

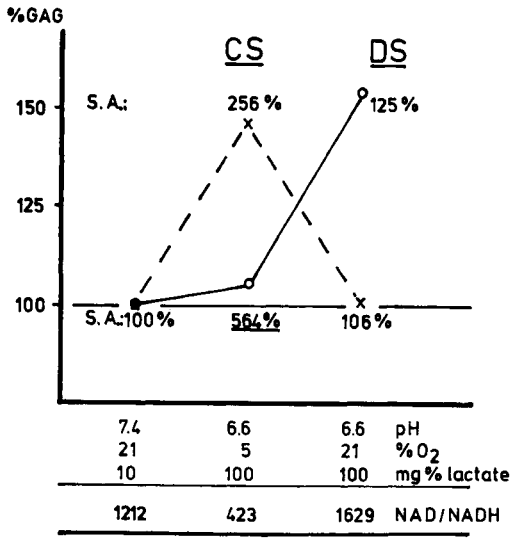

Fig. 1. Comparison of changes of the cytoplasmic NAD/NADH ratios with changes in the concentration and specific activities (^{35}S-sulfate) of CS and DS in proliferating fibroblast cultures in conditions of hypoxic and normoxic glycolysis. ^{35}S-sulfate was applied to the cultures 48 h before harvesting. The GAG of medium plus cells were isolated by means of ethanol precipitation of the PG, by papain digestion, CPC precipitation and fractionation according to SVEJCAR and ROBERTSON (1967). Hexuronic acid was determined after BITTER and MUIR (1962), the ^{35}S incorporation by liquid scintillation counting. — Specific activity and GAG concentration were 100% in normal cultures with *p*H 7 4, 21 vol% O_2 and 10 mg% lactate (results after KITTLICK 1981. Note that the NADH data were gained in 2 vol% O_2, the GAG data, however, in 5 vol% hypoxia.)

Table 2. Ratio of specific activities (^{35}S-sulfate) of DS : CS in preconfluent cultures of embryonic rat fibroblasts (2.2×10^4 cells/cm^2)

	*p*H 7.4	*p*H 6.6
Control	1.05	1.20
Hypoxia (5 vol% O_2)	0.66	1.02
L-lactate (100 mg%)	1.40	1.65
Hypoxia (5% O_2) plus L-lactate (100 mg%)	0.87	2.04

The GAG percentage seems to be increased when the S.A. is medium sized thus allowing a compensatory increase of the GAG concentration by neosynthesis. Therefore the CS percentage may be elevated by "hypoxic glycolysis" but DS percentage by "normoxic glycolysis" (S.A. = 125% compared with 564% under hypoxic conditions).

Discussion

Metabolic heterogeneity of GAG in cell culture and tissues is a well known fact. In bovine heart valves and aortic wall DS has a higher rate of synthesis than CS (KRESSE and BUDDECKE 1970; v. FIGURA et al. 1973). In cultured aortic intimal cells the intracellular degradation is faster for DS than for CS (KRESSE et al. 1975). Hypertension increased the rate of synthesis in the aortic wall for DS but not for CS (KRESSE et al. 1971).

In the past years we observed differently changing ratios in the concentrations and specific activities of DS and CS when the culture conditions had been changed from the normal to an acidic, hypoxic or lactic acid-rich environment (e.g. KITTLICK and NEUPERT 1975; KITTLICK et al. 1976b; KITTLICK 1977b, 1981).

For the proliferating fibroblasts the ratios of S.A. of DS/CS are shown in table 2. In resting cultures the corresponding quotients are changing. They increase with the increase of the H^+ and lactate ion concentrations.

At lowered *p*H, especially when combined with a reducing potential, the turnover rate of DS overcomes clearly that of CS. Therefore an investigation was started to determine the cytoplasmic NAD/NADH ratios under appropriate conditions and to look for a correlation between the relative NADH level and the specific turnover rates.

In the results recorded in table 1, two values are of special interest, the NAD/NADH ratio connected with lactate acidosis at normoxia (*p*H 6.6, 100 mg% lactate, 21 % O_2 = condition of aerobic glycolysis) and the ratio at lactate acidosis with hypoxia (*p*H 6.6, 100 mg% lactate, 2 % O_2 — condition of hypoxic glycolysis). These both ratios, 1629 and 423, differ from one another markedly as considered in "*Results*". The environment of hypoxic glycolysis causes strong increases of the S.A. of CS and, above all, of DS (fig. 1). Under conditions of aerobic glycolysis only the S.A. of DS is moderately increased. These increases in the turnover rates give hints at increased degradative activities, but a regulatory neosynthesis was also recorded.

The NAD/NADH ratio is known to control the GAG precursor synthesis. Thus UDP-glucose-dehydrogenase, UDP-glucuronic acid-dehydrogenase (forming UDP-xylose), UDP-glucose-4′-epimerase and UDP-N-acetyl-glucosamine-4′-epimerase are activated by NAD but nhibited by NADH (Balduini et al. 1973; Phelps 1973; DeLuca et al. 1978, 1983). It was supposed that the low NAD/NADH ratio in growing cells might inhibit the epimerases (Goggins et al. 1972). The NADH inhibition was increaded when the *p*H was decreasing from 8.8 to 7.7 (DeLuca et al. 1978).

In cultures of embryonic rat fibroblasts the GAG total concentration was not influenced by decreased *p*H values, by 100 mg% lactate or 5 vol% O_2 hypoxia (Kittlick et al. 1976a, 1976c; Kittlick and Neupert 1975). It was reduced, however, at *p*H 7.4 by 1 vol% O_2 hypoxia (Kittlick 1977a) or by a combination of 5 vol% O_2 and 100 mg% lactate (Kittlick 1977b, 1981). The relatively high NADH levels produced under those reductive conditions seem not to inhibit the GAG/PG neosynthesis but rather to stimulate the PG breakdown. Synthesis of PG is known to be triggered by proteolytic PG degradation. Therefore the high turnover rates observed when the NADH level is high (fig. 1) indicate high catabolic rates of PG.

Assuming the high S.A. is an indication of primarily elevated PG degradation and the relative GAG concentration is an indication of the level of subsequent PG neosynthesis, we suggest (fig. 1)

— that conditions of hypoxic glycolysis (*p*H 6.6, 100 mg% lactate, 5 vol% O_2) with the lowered NAD/NADH ratio of 423 stimulate the degradation of DS (S.A. = 564 %) more strongly than that of CS (S.A. = 256 %);

— that the specific synthetic capacities are just sufficient to maintain the DS level, but large enough to elevate the CS concentration to 145 %.

Conditions of aerobic glycolysis (NAD/NADH ratio = 1629) do not affect the CS metabolism. The slightly increased, apparently H-ion-activated DS catabolism (S.A. = 125 %) seems here to be overcompensated by neosynthesis of DS-PG and results in an increase to 154 %. As a consequence of these metabolic alterations the GAG distribution pattern is changing.

The syntheses of DS and CS follow a common way until the epimerization at the polymer level at C-5 of glucuronic acid leads from CS to DS (Malmström 1981). The NADH-dependent metabolic differences between both these GAG might be due to the NAD demand for epimerization to L-iduronic acid or due to different core proteins carrying DS- and CS-chains and due to other specific properties of DS-PG causing their favourable proteolytic degradation. There are in question both acidic and NADH-activated proteases, certainly thiol proteases.

KRESSE et al. (1975) discussed the different degradation rates observed in cell culture GAG in the following way:
Extracellular PG are pinocytosed by fibroblast-like cells. The intracellular PG and GAG consist of a small biosynthetic and a large degradative pool the decay of which shows the kinetics of a two-component system. This may be either due to different classes of lysosomes, due to partial location of PG outside the lysosomes or a direct transfer of certain PG from the synthetic to the degradative pool. Furthermore, PG-specific pinocytosis has been observed. A very high rate of pinocytosis was measured for a DS-PG. Proteolytic digestion of the protein core reduced the pinocytotic rate (BUDDECKE 1977; TRUPPE and KRESSE 1978). Structural heterogeneity of PG has been confirmed by GALLAGHER (1977). In our opinion, proteases and proteases with different PG-specificities are a further way ot explain the metabolic heterogeneity of PG and GAG and its alteration in pathological conditions.

The degradation of PG starts with proteolytic breakdown which occurs in physiological conditions mainly intracellularly, but largely extracellularly, e.g., in inflammation. Here enzymes of leukocytic origin participate. In these processes there are involved acid (cathepsin B, D, F) and neutral proteases (cathepsin G, elastase, metalloproteinase, plasmin etc.). The latter group seems to play the most important role in extracellular degradation of PG, whereas acid proteases mediate the intralysosomal breakdown (KEISER 1980).

Investigations have mainly been performed with cartilage PG as the only substrate and with a variety of proteases. The susceptibility of this type of PG to proteolytic attack by, e.g., cathepsin B, D, G and lysosomal elastase differs in so far as different PG-peptides with different numbers of GAG chains are formed (ROUGHLEY and BARRETT 1977). Less is known about the effect of proteoglycanases on different PG types as present in connective tissue other than cartilage or in cultures of fibroblast-like cells.

Recently, we reported on the selective increase of CS in thrombin- or trypsin-treated fibroblast cultures which was accompanied by a more prominent increase of the turnover rate of CS when compared with DS (KITTLICK and NEUPERT 1982a, b). This effect may be due to different degradative and, subsequently, anabolic rates of DS- and CS-PG. In this publication a selective increase of the DS concentration at low cell density was correlated with the conditions of aerobic glycolysis. A decrease of the NAD/NADH ratio does not occur in this case. Therefore the effect on the DS turnover of *p*H lowering, although subsided by lactate abundance, seems to indicate that acid proteases attack DS-PG moderately but not at all CS-PG. As a consequence, there is an overcompensatory increase of DS-PG. This increase mediated by aerobic-glycolytic conditions is correlated with tissue maturation (for literature see "*Introduction*").

Among the proteases mentioned above, cathepsin B has functional thiol groups. This enzyme is well characterized and occurs within all mammalian cells. Extracellular lysosomal thiol proteases are mainly derived from neoplastic cells (KATUNUMA and KOMINAMI 1983). Another thiol protease which degrades PG has recently been described in pseudoxanthoma elasticum fibroblasts (*p*H optimum 6.0). It is Zn-dependent. In normal fibroblasts, however, a Ca-dependent cysteine proteoglycanase was found (GORDON et al. 1983).

Lysosomal SH-proteases take part in limited proteolysis, in intracellular regulation and in protein degradation. They are activated by NADPH or NADH via glutathione (GSH). A natural inhibitor of these enzymes is likewise activated by GSH (KATUNUMA and KOMINANI 1983). In tumor cells an inhibitor with reactive thiol groups has been found which, by thiol-disulfide exchange, forms intermolecular disulfide bonds and thus provides latent forms of trypsin, chymotrypsin, elastase etc. (STEVEN et al. 1980). Further examples of this newly recognized principle of enzymatic regulation have been published (FRANCIS and BALLARD 1980; MANNERVIK and AXELSSON 1980; TSCHECHE and MCCARTNEY 1981, 1982). Therefore the redox potential may be an effective means of precise control of the balance between active and inhibited states of thiol proteases. NADH may cause inhibition or activation of appropriate enzymes containing SH- and S-S-groups.

In the light of the facts considered we suppose that the NAD/NADH ratio determines the activity of PG-degrading enzymes. As a consequence of different PG degradation and different

compensatory neosynthesis the DS/CS ratio changes. At low cell density, as discussed here, the CS concentration increases in conditions of hypoxic glycolysis. The increase of CS corresponds with proliferative and chronic inflammatory processes (literature see "*Introduction*") An improved supply with oxygen in these cases might be able to reduce anaerobic glycolysis, CS concentration and continued fibroblast proliferation, but to advance DS and tissue maturation.

Generally it appears that different proteases, acidic, neutral and NADH-dependent, are involved in modifying the pattern of proteoglycans thus determining the fate of the tissues.

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