Oxidative stress in chronic lymphoedema

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Summary

Background: Chronic lymphoedema is one of the most frequent and debilitating complications after surgical and radiological tumour treatment. Prevention and therapy of lymphoedema is therefore an important problem of the rehabilitation of those patients.

Aim: To investigate whether chronic lymphoedema results in increased oxidative stress.

Design: Prospective case-control study.

Methods: We obtained venous blood samples from patients ($n = 38$) with chronic lymphoedema and determined biomarkers of prooxidative reactions and of antioxidative defense system in the erythrocytes or blood plasma: reduced and oxidized glutathione (GSH and GSSG), and lipid peroxidation products such as malondialdehyde (MDA) and 4-hydroxynonenal (HNE). Healthy volunteers ($n = 90$) and patients who had undergone surgical and/or radiotherapeutic treatment of tumours without consequent lymphoedema ($n = 20$) acted as controls.

Results: The blood of patients with chronic lymphoedema contained lower concentrations of GSH and higher levels of GSSG and of MDA and HNE, compared with the control group. MDA was increased by about three-fold in the serum of the lymphoedema patients. Accelerated free radical formation and lipid peroxidation processes were further demonstrated by the liberation of MDA and HNE into the blood serum after manual lymph drainage.

Discussion: Our data demonstrate enhanced formation of reactive oxygen species (ROS) and accelerated lipid peroxidation processes in chronic lymphoedematous tissue. The strengthening of antioxidative defense mechanisms could be useful in the therapy of chronic lymphoedema.

Introduction

Chronic lymphoedema affects an estimated 200 million people worldwide and is currently incurable. The different forms of lymphoedema are generally divided into so-called primary or congenital lymphoedema, and acquired or secondary lymphoedema. In tropical regions, millions of cases of lymphoedema arise from infection of the lymphatics by filarial parasites. In Europe and Northern America, the main cause of chronic lymphoedema is cancer and cancer treatment, making the prevention and therapy of chronic lymphoedema an important problem of cancer rehabilitation.[1–8]

Schuenemann and Willich intensively investigated the incidence of lymphoedema of the arm in patients with breast cancer, treated by either surgery or radiotherapy, between 1972 and 1995. The authors observed 1405 cases of arm lymphoedema after treatment of 5868 cases of breast cancer, i.e. 24%.[9] Often chronic lymphoedema

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occurs in surgically treated patients with carcinomas of the prostate and the sigma, with seminomas and other tumours of the genital system, too. Lymphoedema may affect either one or both arms or legs or the head.

Chronic lymphoedema is thus a disease of high clinical relevance. On the other hand, lymphoedema may be considered as a 'pathological' model of ischaemia-reperfusion-related tissue injury. Lymphoedema develops due to a limited transport capacity of lymph vessels, leading to increased interstitial volume, increased interstitial pressure and increased interstitial osmotic pressure. This last is due to the fact that the lymphoedema is a protein-rich oedema, in contrast to other kinds, such as oedema related to heart insufficiency, renal failure or hepatic diseases. The most important pathological changes of lymph vessels and interstitial tissue due to increased volume, pressure and osmotic pressure in interstitial space are: dilatation of lymph capillaries, morphological and functional damage of endothelial cells, sclerosis of collector lymphatics, and reduced number of smooth muscle cells of lymphatics.[10–12] Under these conditions, serious regional oxygen deficiency followed by reperfusion periods can occur in lymphoedematous tissue. Furthermore, polymorphonuclear leukocytes (PMNL) migrate into the lymphoedematous region, and secondary inflammatory processes are observed.[10,11] From those changes we postulated an increased formation of reactive oxygen species and accelerated lipid peroxidation processes in lymph vessel and interstitial tissue.

The aim of this study was to investigate whether chronic lymphoedema is connected with increased oxidative stress. We therefore compared the concentrations of reduced and oxidized glutathione (GSH and GSSG) of erythrocytes and concentrations of malondialdehyde (MDA) and 4-hydroxynonenal (HNE) in the blood plasma of patients with chronic lymphoedema, with those of healthy controls.

Methods

Biochemical changes (glutathione status of red blood cells, MDA and HNE of blood plasma), and clinical chemical and haematological parameters were studied in 38 patients (31 women, 7 men) with lymphoedema following surgical or surgical and radiological treatment of tumours. The surgical operation or last radiation was at least one and a half years ago. Overall, 20 patients suffered from leg lymphoedema, 13 from arm lymphoedema

and five from head lymphoedema. The mean age was 50.6 ± 13.6 years (mean $\pm$ SE). At the time of measurements, all patients were without tumour recurrence or detectable metastases. The main control group consisted of 90 healthy people (72 women, 18 men). The mean age of the controls was 48.1 ± 15.7 years (mean $\pm$ SE). All the patients and controls who were investigated did not take antioxidants such as vitamin E or ascorbic acid, and did not suffer from acute infectious diseases (to exclude the interference of those factors on the balance between pro-oxidants and antioxidants). An additional control group was recruited from 20 patients with surgical and/or radiotherapeutic treatment of tumours (prostata, uterine cervix, breast, ovarium) but without consequent lymphoedema. This patient group was used to exclude any long-lasting changes in oxidative stress parameters due to the tumour itself or the tumour treatment. The mean age of these additional controls was 48.2 ± 14.6 years; 16 were female and four were male. The time interval between surgical operation/last radiation and measurement of oxidative stress parameters was at least two years, mean 4.0 ± 2.7 years.

In eight female patients with chronic arm lymphoedema, the biochemical, clinical chemical and haematological parameters were measured before, during and after the so-called complex physical therapy. The first blood sample was taken immediately before the start of manual lymph drainage, the second sample was taken at the end of drainage, i.e. after 30 to 35 min of treatment, and the third sample was taken after completing the compression bandaging of the lymphoedematous extremity, i.e. after a further 10 min. The total duration of the complex physical therapy (CPT) including manual lymph drainage and compression was about 40–45 min. This limited patient group was only used in the data shown in Table 2. For all other measurements, all patients were investigated.

Venous blood samples were taken from the arm vein; in the case of patients with arm lymphoedema, from a vein of the contralateral arm, to avoid mechanical injury. Sodium citrate was added to the blood samples to prevent coagulation. Plasma was separated from blood cells by centrifugation for analysis of MDA and HNE. The data of patients with chronic lymphoedema were obtained at the time point of the first consultation in the clinic (lymphology group) and before the first complex physical therapy.

For analysis of GSH and GSSG concentrations, total blood was drawn with ice-cold metaphosphoric acid and kept at 4 °C. Samples were centrifuged for 10 min at 1200 g. Supernatants were

collected and separated into two aliquots for the GSH and GSSG measurements.[13] GSH was assayed by means of Ellman's reagent (DTNB).[14] GSSG was determined fluorimetrically after addition of o-phthaldialdehyde.[15] GSH autoxidation was prevented by addition of 50 mM N-ethylmaleimide (NEM).

MDA was measured by reaction with thiobarbituric acid (TBA) and HPLC separation of the MDA-TBA conjugate.[16]

Measurement of HNE was done by modifying this aldehyde with dinitrophenylhydrazine, TLC separation of dinitrophenylhydrazones into three groups and finally the isocratic separation of dinitrophenylhydrazine-derivatives of 4-hydroxyalkenals.[17–19] For HPLC analysis, a methanol:water mixture (4:1, v:v) was used as eluent. The detection wavelength was 378 nm. HNE standard was prepared from the diacetal, which was stored as a solution in chloroform at $-20\ °C$.

After normality of data distribution had been determined, differences between patients with chronic lymphoedema and controls were tested by analysis of variance. Pairwise comparisons were done using the independent two-sided t-test, p values < 0.05 being regarded as significant.

Results

Table 1 shows clinical chemical and hematological parameters of patients and controls. There were only a few differences between the two groups. LDH and CK activities were increased in the serum of patients, and mean alanine aminotransferase activity was increased by about 25%, but not significantly. Mean erythrocyte sedimentation rate was increased in patients, due to typical and well-known inflammatory reactions in lymphoedematous regions. The number of leukocytes was also slightly increased in the blood of patients. The mean value of iron level was decreased (data not shown), but not significantly.

The glutathione status of blood and the aldehydic lipid peroxidation products of serum were measured under steady-state conditions, before the start of complex physical therapy. The erythrocytes of patients with lymphoedema had reduced GSH concentrations (Figure 1). GSH was decreased by about 20% from that of the controls, with high significance. GSH in the additional control group (tumour patients without lymphoedema) was not different from that in healthy controls. Furthermore, erythrocytic GSSG of the patients was about double that of either control group (Figure 1). Decreased

Table 1 Clinical chemical and hematological parameters of patients with secondary chronic lymphoedema as compared with healthy controls

	Patients	Healthy controls	p
Haematocrit (%)	41.1 ± 3.7	42.0 ± 4.0	NS
Haemoglobin (mmol/l)	8.636 ± 0.826	8.80 ± 0.93	NS
Erythrocytes (Gpt/µl)	4.62 ± 0.45	4.7 ± 0.4	NS
Leukocytes (Gpt/ml)	6.69 ± 1.81	6.1 ± 1.3	0.05
Thrombocytes (Gpt/ml)	265 ± 81	254 ± 57	NS
MCV (fl)	88.5 ± 4.5	90.5 ± 6.2	NS
ESR (mm/h)	19.9 ± 16.3	10.2 ± 5.1	0.05
Bilirubin (µM)	9.9 ± 4.2	10.4 ± 7.0	NS
Cholesterol (mM)	5.75 ± 1.20	5.4 ± 0.5	NS
Triglycerides (mM)	1.42 ± 0.61	1.5 ± 0.6	NS
Uric acid (µM)	286 ± 49	269 ± 57	0.05
Protein (g/l)	72.0 ± 2.1	72.0 ± 4.0	NS
Albumin (%)	60.9 ± 5.4	61.5 ± 6.3	NS
Alpha 1 (%)	3.2 ± 0.9	3.5 ± 0.5	NS
Alpha 2 (%)	7.9 ± 1.6	8.3 ± 1.3	NS
Beta (%)	10.7 ± 2.3	10.5 ± 1.5	NS
Gamma (%)	17.0 ± 4.1	16.1 ± 3.2	NS
ALAT (katal)	0.41 ± 0.25	0.32 ± 0.20	NS
ASAT (katal)	0.30 ± 0.07	0.27 ± 0.10	NS
AP (U/l)	125.8 ± 56.8	105.9 ± 31.1	NS
LDH (katal)	4.70 ± 1.90	2.6 ± 0.4	0.002
CK (katal)	$2.87 \pm 0\ 80$	1.90 ± 0.41	0.01

Values are means $\pm$ SE. Values of the additional control group (treated tumour patients without lymphoedema) were not different from those of healthy controls, e.g. haematocrit 42.3 ± 1.6; haemoglobin 8.92 ± 0.46; erythrocytes 4.60 ± 0.31, leukocytes 6.2 ± 0.9, thrombocytes 255 ± 39; ESR 9.4 ± 3.7, etc. ESR, Erythrocyte sedimentation rate; ALAT, alanine aminotransferase; ASAT, aspartate aminotransferase; AP, alkaline phosphatase; LDH, lactate dehydrogenase; CK, creatine kinase.

GSH and increased GSSG led to a threefold-higher glutathione ratio (0.088 ± 0.012 vs. 0.036 ± 0.014) calculated on the basis of sulfhydryl units, i.e. 2 GSSG:[GSH + 2 GSSG]. This increase is an indicator of oxidative stress in the erythrocytes of patients with chronic lymphoedema.

Figure 2 shows the increased levels of cytotoxic aldehydic lipid peroxidation (LPO) products in the serum of the patients. The level of HNE, a highly specific LPO marker, is twofold higher in the serum of patients in comparison with the physiological HNE level in the healthy control group and in the additional control group. The more common but less specific aldehydic LPO marker MDA was about threefold higher in the serum of

Table 2 Aldehydic lipid peroxidation products MDA and HNE during complex physical therapy of the lymphoedematous arm of female patients with chronic lymphoedema

Parameter	Before (0 min)	After drainage (30 min)	After compression (40 min)
Serum MDA (nmol/ml)	0.75	0.87*	0.90*
Serum HNE (nmol/ml)	0.13	0.30*	0.19*
Haematocrit (%)	40.9	37.9*	39.0
Haemoglobin (mM)	8.7	8.1*	8.6
Erythrocytes (Gpt/µl)	4.73	4.41*	4.58
Leukocytes (Gpt/ml)	6.6	6.8	7.3*
Thrombocytes (Gpt/ml)	265	234*	246
MCV (fl)	86.4	85.9	86.6

Complex physical therapy (CPT) includes the manual lymph drainage and the following compression. The drainage takes about 30–35 min, the compression about 10 min, i.e. total 40–45 min. Measurements before, during and after CPT were performed in eight patients. Values are means. *Significant change in comparison with the initial value (0 min), $p < 0.05$.

the lymphoedema patients compared with the two control groups.

Figure 3 shows the inverse interrelationship between MDA and GSH. A high serum MDA concentration is connected with a lowered erythrocytic GSH level.

Manual lymphatic drainage is the main component of the complex physical therapy (CPT). The CPT as the common therapeutic procedure for patients with chronic lymphoedema includes manual drainage, which induces the transport

of interstitial fluid via lymphatic system to the thoracic duct and into the circulating blood. The compression that follows reduces the new formation of interstitial fluid. Table 2 shows that manual lymphatic drainage led to an increase of MDA and HNE in the circulating blood serum, in 8 female patients with arm lymphoedema after surgical treatment of breast cancer. MDA increased by about 20% during the lymph drainage of the oedematous arm; HNE level increased by ~2.5-fold. The MDA increase continued after the compression of the oedematous arm following drainage, whereas the HNE increase was only short-lived. In Table 2, haematological measurements are given as reference data to demonstrate the dilution of circulating blood volume by effective drainage therapy (decreased haematocrit, etc.).

Discussion

The GSH, GSSG, MDA, and HNE values of our healthy controls and of our treated tumour patients without lymphoedema were in the range of normal values in previous studies.[13] The decreased GSH and the increased GSSG, MDA, and HNE in our lymphoedema patients confirms the hypothesis that ROS generation and lipid peroxidation processes are accelerated in chronic lymphoedematous tissue. The resulting pro-oxidative changes measured in blood and serum are in accordance with findings of Ohkuma on increased levels of lipoperoxides in the dermis of lymphoedema.[20,21]

Using specific methods of MDA and HNE quantification, we found a doubled level of serum HNE and even a threefold increased level of serum MDA in chronic lymphoedema. Clearly, the increased ROS formation and LPO in the lymphoedematous tissue is so large that it can be measured in the circulating blood. Only in a few other diseases have such drastic increases in MDA and HNE been described, e.g. end-stage renal failure.[22] The fact that changes in the glutathione system could be measured even in circulating red blood cells argues for a drastic increase in the generation of ROS in lymphoedematous tissue.

The sources of this accelerated ROS formation and lipid peroxidation in chronic lymphoedema are likely to be periods of regional hypoxia/reoxygenation, and the effects of PMNL migrating into the region. Kurtel *et al.* compared formation of thiobarbituric-acid-reactive substances (an MDA-like parameter) by lymph and serum in a model with 2,2'-azobis(2-amidinopropane)hydrochloride, which yields peroxyl radicals, and in a model with
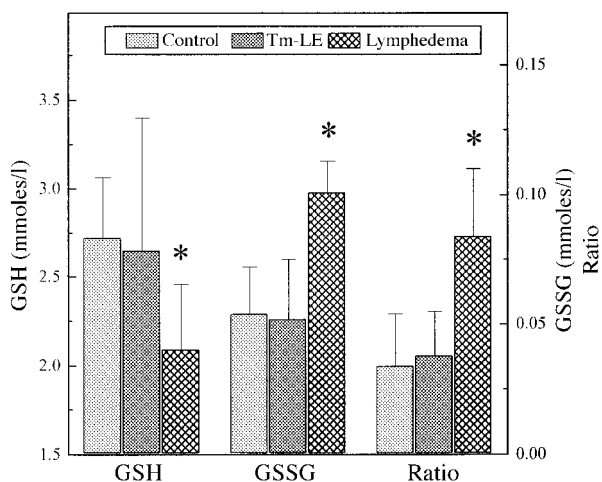

Figure 1. GSH, GSSG and glutathione ratio in erythrocytes of patients with chronic lymphoedema ($n = 38$), healthy controls ($n = 90$) and patients with treated tumour without lymphoedema (Tm-LE) ($n = 20$). Values are means ± SE (*$p < 0.05$).
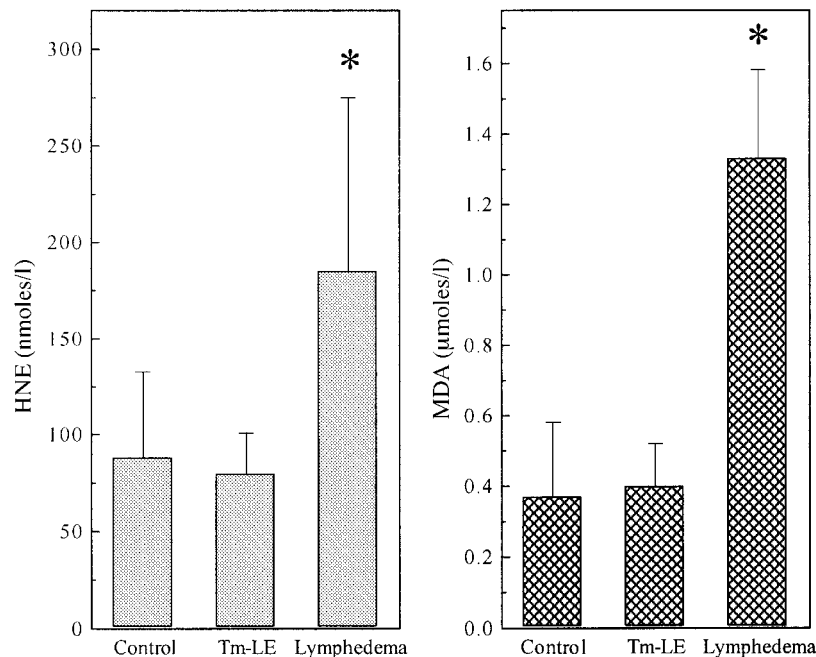
Figure 2. Lipid peroxidation markers MDA and HNE in blood plasma of patients with chronic lymphoedema and controls. HNE is a very specific marker of lipid peroxidation and is formed from Ω-6 polyunsaturated fatty acids. $n = 38$ for patients with lymphoedema; $n = 90$ for healthy controls and $n = 20$ for Tm-LE (tumour without lymphoedema); mean ± SE (*$p < 0.05$).

activated PMNL. In both models, plasma/serum was more resistant to lipid peroxidation than was interstitial fluid, on the basis of their different composition as regards peroxidizable substrates and antioxidative compounds.[23]
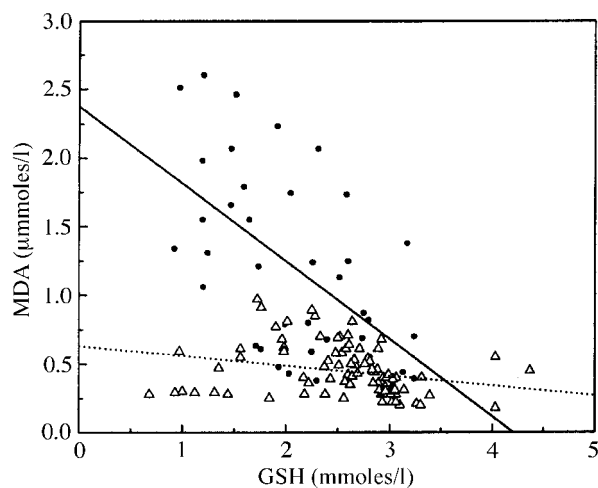

Figure 3. MDA serum levels in relation to GSH. GSH is one of the most important hydrophilic antioxidants and the compound with the highest sulfhydryl concentration in red blood cells. Data from surgically treated tumour patients with secondary chronic lymphoedema ($n = 38$) (●) and from healthy controls ($n = 90$) (△). Significance of the correlation between MDA and GSH was $p = 0.00016$ for the lymphoedema patients and $p = 0.012$ for the controls.

The concentration of LPO products is always the result of the rates of formation and degradation of these products. Taking into account the high reactivity and the rapid metabolism of aldehydic LPO products demonstrated for different cell types and tissues,[24,25] an accumulation of MDA and HNE in the serum is always due to very high generation rates of these aldehydes. Differences in the dynamics of MDA and HNE levels can be due to different degradation rates for both cytotoxic aldehydes.

In fact, the levels of aldehyde LPO products seem to depend on the total volume of lymphoedema fluid. The volume of a leg lymphoedema in many cases is about 4–5 l (in our study 4.1 ± 2.2 l), the volume of an arm lymphoedema in the range of 0.5–1 l (in our study 0.59 ± 0.55 l). Aldehyde levels are higher in patients with leg lymphoedema in comparison with patients with arm lymphoedema. Obviously in patients with a higher lymphoedema volume, aldehyde release into the blood circulation is increased, leading to higher aldehyde levels in the blood and serum. One can conclude that the higher the volume of the lymphoedema fluid, the higher the oxidative stress for the whole organism.

The results of our studies indicate that the oxidative stress related changes are a consequence of the lymphoedema, since the control group that had treated tumours but no lymphoedema had

data comparable to the healthy controls. We conclude that the changes in the oxidative stress parameters are related to the lymphoedema.

The 'consumption' of antioxidants in the serum and blood such as GSH underlines the high formation rate of ROS and LPO products such as MDA (Figure 3). Aldehydic LPO products react rapidly with GSH, catalysed by glutathione transferases, which are ubiquitous enzymes, and particularly rich in the liver.[26]

It is already known from the literature that ROS are able to disturb lymphatic contractions.[27,28] The oxidative stress measured in our study, therefore, may be an important pathophysiological factor in the progression of the disease. The toxic effects of aldehydic lipid peroxidation products such as MDA and HNE have been described in detail.[26] The binding of HNE to sulfhydryl and amino groups of proteins and peptides leads to the inhibition of enzyme activities.[29–33] There are data on the modulation of fibroblast proliferation by oxygen free radicals and lipid peroxidation products, on the stimulation of collagen α_1(I) gene expression in association with lipid peroxidation and on the interrelationships between oxidative stress and fibrogenesis.[34] On this basis, it seems reasonable to suggest an influence of increased MDA and HNE on fibrosis and sclerosis as the typical changes in chronic lymphoedema.

On the basis of our measurements, we propose that the reduction of ROS formation or the strengthening of antioxidative defense mechanisms could supplement the complex physical therapy[35,36] as the main therapeutic procedure in chronic lymphoedema. These data suggest a need for clinical studies on the administration of anti-oxidative protective substances in this so far incurable disease.

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