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EFFECT OF COMPLEX DECONGESTIVE PHYSIOTHERAPY ON GENE EXPRESSION FOR THE INFLAMMATORY RESPONSE IN PERIPHERAL LYMPHEDEMA

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ABSTRACT

Complex decongestive physiotherapy (CDP), consisting of manual lymph drainage, compression bandaging, remedial exercises and skin care, mobilizes accumulated edema fluid and increases lymph flow. On the other hand, it also has a beneficial therapeutic effect on fibrosclerosis. Because little is known of its possible mode of action on a molecular level, this preliminary study evaluated CDP in patients with peripheral leg lymphedema as to the potential role of gene expression in the inflammatory response. The quantitative expression of genes for CD14, interferon-γ receptor (IFNγR), tumor necrosis factor-α (TNFα), integrin $\alpha_4\beta_1$ (VLA-4), tumor necrosis factor receptor p55 (TNFR1) and CD44 (standard form) was examined in 9 patients with primary or secondary leg lymphedema before and after phase 1 of CDP. Overall, there was a decrease of expression of these pro-inflammatory genes after CDP, suggesting that biologic mechanisms implicated in the inflammatory cascades in other disorders are also involved in the fibrosclerotic reactivity in lymphedema. However, whereas each patient acted as his or her own control before and after CDP, gene expression in normal patients and normal limbs before and after CDP needs to be examined before the full meaning of these observations can be understood.

With lymphangiopathies and/or lymphadenopathies, lymphedema is not exclusively a disorder of increased tissue fluid content. As a result of the proliferation of connective tissue and matrix proteoglycans, even in the early stages of lymphedema, there is soft tissue transformation and altered migration of circulating cell populations. Up to now, molecular mechanisms and their possible role in lymphostatic tissue transformation are largely unknown. Whereas there has been considerable research on gene expression during acute inflammation and on inflammatory processes in other chronic disorders, little or none on this issue exists in the condition of lymphedema. Nevertheless, it may be assumed that the general rules of cell intercommunication and inflammation such as the interaction of cytokines and growth factors with their respective receptors should also apply to lymphedema. As the ongoing migration of mononuclear cells into tissues ("cellular response") is likely impaired with lymphostasis (1), we focused on the activation of the monocyte, its adhesion to blood vascular endothelium and its migration through the interstitium (now as a macrophage). We chose to examine the following genes: the cytokine CD14 and the interferon-γ (IFNγR), which activate monocytes; CD14, tumor necrosis factor-α (TNFα) and the tumor necrosis factor receptor p55 (TNFR1),

which regulate adhesion of monocytes to the blood vascular endothelium (2,3); the integrin $\alpha_4\beta_1$ (VLA-4), which regulates adhesion of leukocytes to the blood vascular endothelium (4); and CD44 as a receptor for hyaluronan (5), which acts as a scaffold possibly influencing migration of leukocytes through the interstitium.

Because complex decongestive physiotherapy (CDP) has been effective in softening connective tissue, we aimed in this pilot study to examine molecular mechanisms that may play a role in connective tissue proliferation by quantifying gene expression of CD14, IFNγR, TNFα, VLA-4, TNFR1, and CD44 before and after phase I CDP.

CLINICAL SUBJECTS

Nine patients (7 women, 2 men; average age: 53.7±5 years) with lymphedema of the leg (primary or secondary) in stage II or III according to Brunner (6) voluntarily participated in this study. The following disorders were excluded by appropriate clinical and laboratory studies: other forms of coexistent edema, skin diseases such as psoriasis or atopic dermatitis, postphlebitic syndrome or chronic venous insufficiency, neurological disorders or neuropathies, rheumatic diseases, acute erysipelas, recurrent cancer.

Complex Decongestive Physiotherapy (CDP)

Phase I of CDP consisting of manual lymph drainage and compression bandaging (Comprilan™, Tricofix™, Beiersdorf AG, Hamburg, Germany) was performed twice daily accompanied by remedial exercises and skin care. The lymphological compressive bandages were applied for 20 hours each day. Before the start of CDP and at the end of phase I (average 24.5±3 days), venous whole blood samples were preserved in EDTA. Leg volume measurements (truncated cone formula) and direct palpation were used as a gauge of therapeutic improvement in edema.

MEASUREMENTS

Quantitative Analysis of Gene Expression

RNA isolation from cooled EDTA whole blood was begun within 1 hour of sampling. Total cellular RNA was extracted from unseparated, pelleted cells using monophasic phenol/guanidine-HCl at pH 6.5. After repeated isopropanol/ethanol precipitations, the isolated RNA was rendered DNA-free by treatment with RQ1-DNase (Promega), and purified by RNeasy (Qiagen). Reverse transcription was performed with random-hexamer primer and Superscript II RTase (Life Technologies). Specific gene transcripts of CD14 (EMBL AC M86511), IFNγR (EMBL AC J03143), TNFα (EMBL AC M10988), VLA-4 (EMBL AC 16983), TNFR-1 (EMBL AC M63121), and CD44s (EMBL AC L05407-L05424, exons 1-5, 15-18) and ß-actin and the reticulocyte-specific hexokinase (HKR) were determined by polymerase chain reaction (PCR) in a manner of reciprocal complementary titration as described by Nicoletti et al (7). This method ensures precise relative quantitation in pairwise comparisons of samples. With high precision due to mathematical regression, this technique ensures high reliability of data that is not uniformly achieved with conventional PCR applying single tube determinations of single samples. Hot-start PCRs with recombinant Taq-DNA-polymerase (Life Technologies) were processed in 10 µl assays in a GeneAmp 2400 thermocycler (PE Biosystems) using cycling protocols validated and optimized separately for the various gene transcripts. PCR was terminated clearly within linear log-phase of amplification as verified experimentally. Quantification of the PCR signals was performed by high-resolution digital video measurements of the fluorescent DNA-fragment bands in agarose gel after electrophoresis and after staining with ethidium bromide until stoichiometric balance of dyeing. The CCD video signal was standardized by a 1000 bp DNA quantitation

TABLE 1
Demographics and Leg Volume Reduction During Phase I of Complex Decongestive Therapy

Patient	Sex	Age	Primary/ Secondary	Clinical Stage	Localization	Volume Reduction Right [ml]	Volume Reduction Left [ml]
1	male	73	secondary	II	left	—	-1600
2	female	68	primary	III	left, right	-3000	-4400
3	female	52	primary	III	left, right	-8170	-10641
4	female	37	primary	II	left, right	-900	-840
5	female	43	primary	III	left, right	-1100	-812
6	male	69	primary	left: III right: II	left, right	-700	-750
7	female	68	primary	II	left, right	-1170	-1300
8	female	49	secondary	III	right	-1700	—
9	female	55	secondary	II	left, right	-450	-1000

standard (Advanced Biotechnologies). Raw data were computerized by RFLPScan software (Scanalytics) and normalized to the constant expression of the "housekeeping" gene ß-actin. Because unfractionated blood was used and therefore the proportion of reticulocytes and leukocytes may vary at both examination times, the data were additionally corrected for HKR expression. This procedure ensured that the pre and post comparisons were made with equal leukocyte proportions.

RESULTS

CDP was tolerated by all 9 patients without complications. During the inpatient stay at the clinic, no concurrent diseases occurred. Volume reduction of the swollen leg on average was 4200±3100 ml (*Table 1*), and tissue induration softened. The expression of examined genes in the leukocytes, namely CD14, IFNγR, TNFα, VLA-4, TNFR-1, and CD44s, was consistently reduced (*Table 2*). In one patient (#8), where gene expression was not lowered, she had previously developed radiation colitis after treatment of uterine carcinoma 13 years earlier. Although the colitis was quiescent and she was in tumor remission, it remains possible that ongoing smoldering chronic inflammation contributed to consistent elevation in gene expression. *Table 2* shows the mean ± SD of gene expressions before and after CDP. No direct correlation between the decrease of gene expression and characteristics of lymphedema such as primary or secondary in origin, clinical stage, or volume reduction during CDP was observed.

DISCUSSION

In this pilot study, 9 patients with leg lymphedema (stages II and III) were examined. Phase I of CDP markedly reduced leg volume and softened the connective tissue. We hypothesized that in lymphedema, CDP interferes with the autocrine and paracrine local responses promoting proliferation of macrophages and fibroblasts, which continuously secrete cytokines, growth factors and related receptors.

CD14 helps initiate the inflammatory

TABLE 2
Relative Gene Expression After Phase I of Complex Decongestive Physiotherapy Compared to 100% Before onset of Therapy. Individual Values (% change) for Each Patient is Shown as well as the Mean and Standard Deviation (SD).

Patient	CD14 (%)	IFNγR (%)	TNF-α (%)	VLA-4 (%)	TNFR1 (%)	CD44 (%)
1	▼45	▼42	127	▼73	▼39	▼56
2	▼56	▼68	▼68	▼0	▼41	▼44
3	▼43	▼41	▼53	▼38	▼43	▼41
4	▼76	▼76	▼52	▼61	▼49	▼57
5	▼43	▼55	▼43	▼30	▼26	▼31
6	▼28	▼32	▼32	▼32	▼26	▼26
7	▼26	▼37	▼37	▼27	▼27	▼21
8	92	165	▼65	92	92	▼82
9	▼38	▼60	▼38	▼38	▼38	▼32
Mean ▼	50	64	57	49	42	42
(Mean w/o #8)▼	44	51	56	44	36	37
SD	22	41	29	22	20	20

cascade by activating monocytes and interacting with blood vascular endothelial cells. Although its upregulation had first been noted in sepsis, recent data suggest that CD14 is regulated on the transcription level independently of bacteremia by other cytokines and by hormones (8-11). Our findings suggest that CD14 is also involved in the inflammatory process associated with lymphedema although from these data we cannot discern whether this effect derives from monocyte activation or from interaction with the microvascular endothelium or both factors.

IFNγR, which has been implicated in the activation of leukocytes, also sharply decreased after CDP.

TNFR1 has an important role (and TNFα an exclusive role) in the infiltration of leukocytes into the interstitium (2). During CDP, TNFR1 and TNFα were down-regulated, suggesting their playing a major role in fibrosclerosis associated with lymphedema.

TNFα is involved in the induction of integrins such as VLA-4, which couples monocytes to the walls of blood vessels and facilitates their migration into tissues (4) and was reduced by CDP as was the expression of VLA-4.

CD44 glycoprotein has a profound homeostatic effect on the intracellular matrix as a receptor for hyaluronan (5), as also shown with regard to edema development in an animal model (12). A reduction in CD44 expression after CDP suggests an important role for this gene in the persistence of lymphedema.

Taken together, these findings suggest that proinflammatory cytokines and receptors for growth factors are upregulated in patients with primary and secondary lymphedema and become downregulated by CDP. It seems reasonable that the development of fibrosclerotic tissue in patients with lymphedema or lymphangiopathies associated with lymph

stasis may derive from dysregulation of these molecular mechanisms. Perhaps this derangement (over-expression of the genes for CD14, TNFα, TNFR1, IFNγR, VLA-4) is the first step towards activation of monocytes and their interaction with endothelial cells of blood and lymph capillaries in lymphedema, which then has a profound effect on both tissue fibroblasts and extracellular matrix with subsequent deposition of collagen fibers. Ultimately, the proteoglycans (expression of hyaluronan receptor CD44) are also altered, which is pivotal for homeostatic regulation of tissue water and protein content.

Further investigations, including gene expression in normal patients and the effects of CDP in normal limbs, however, need to be pursued to determine the true significance of these findings.

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