Estrogen receptors in giant cell arteritis. 
An immunocytochemical, Western blot and RT-PCR study

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Abstract
Objective
Giant cell arteritis (GCA) is a chronic form of vasculitis which predominantly affects women over 50 years of age. The aim of this study was to analyse the presence of estrogen receptor α (ER) in the temporal arteries of patients with GCA.

Methods
Inflamed temporal artery biopsies from 43 GCA patients were stained with monoclonal antibodies to two different segments of the ER and compared with non-inflamed arteries from age- and sex-matched controls who had not received a clinical diagnosis of GCA. The protein that was extracted from 4 GCA-positive biopsies and 4 non-GCA controls was analysed using the Western blot method with a monoclonal antibody to ER. Reverse transcriptase-polymerase chain reaction (RT-PCR) analysis using primer pairs specific to ER-cDNA was performed on the total RNA from 4 GCA-positive biopsies and 4 non-GCA controls.

Results
The inflamed arteries expressed distinct cytoplasmic immunoreactivity to ER in activated mononuclear inflammatory cells and in giant cells. Biopsies from GCA patients and controls displayed cytoplasmic ER positivity in smooth muscle cells. Western blot analysis revealed two bands corresponding to approximately 64 and 54 kDa, respectively, in the inflamed arteries and controls. In the inflamed biopsies and non-GCA controls, RT-PCR analysis revealed a strong band corresponding to approximately 670 bp, as expected, and a weaker band corresponding to approximately 440 bp.

Conclusion
In inflamed arteries from GCA patients, smooth muscle cells, activated mononuclear inflammatory cells and giant cells express cytoplasmic ER. Non-inflamed control arteries also express cytoplasmic ER in smooth muscle cells. The accumulation of cytoplasmic ER may suggest the involvement of estrogen not only in GCA but also in normal vascular aging. The results justify further investigations into the pathogenetic roles of estrogen metabolism in GCA.

Key words
Giant cell arteritis, estrogen receptors, vascular smooth muscle cells, inflammation.
Estrogen receptors in giant cell arteritis / V. Petursdottir et al.

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Introduction

Giant cell arteritis (GCA) is a chronic form of vasculitis which predominantly affects women over 50 years of age. Its etiology and pathogenesis are incompletely understood, but they appear to be multifactorial. Genetic factors are of relevance; the disease is more common in patients expressing the histocompatibility antigen HLA-DR4 (1) and in Caucasians (2). Arterial atrophy, which should not be confused with post-inflammatory scarring, appears to play a significant role in the pathogenesis of GCA (3). Immunological analysis of the inflammatory infiltrate in the vessel wall indicates the local activation of T lymphocytes (4), which supports the theory that cell-mediated immunity is involved in GCA. When studying the V-beta families of the T cell infiltrate with flow cytometry, Schaufelberger et al. found that the infiltrating lymphocytes are polyclonal (5). Weyand et al. demonstrated, however, the identical clonal expansion of a small proportion of the T lymphocytes in separate segments of the same artery, which indicates antigen stimulation (6). It has been speculated that various infections could trigger the inflammatory process in GCA (7). Infectious agents have not, however, been isolated from arterial tissue and no viral inclusions have been found in the lesions. Could the fact that GCA is far more common in women than in men and is rarely seen before the age of 50 be related to differences in sex hormone metabolism? The estrogen production of pre-menopausal women is cyclic, with a higher secretion rate than that in men. Moreover, menopause involves a major change in estrogen metabolism. Functioning estrogen receptors (ER) have been detected in cultured human vascular smooth muscle cells (8), indicating that estrogen may be involved in vessel-wall metabolism. Moreover, a number of studies indicate that estrogen plays an important role in immunology (9-11). No morphological studies regarding the role of estrogen in GCA have so far been presented. One way to investigate estrogen metabolism is to study the ER, the receptor through which estrogen modifies gene transcription. The aim of the present investigation was to analyse the presence of ERα in the arteries of patients with GCA using immunocytochemistry, Western blotting and RT-PCR.

Material and methods

Immunocytochemical study

Forty-three temporal artery biopsies given a histopathological diagnosis of GCA were collected from the datafiles at the Department of Pathology, Sahlgrenska University Hospital. The patients underwent temporal artery biopsy due to suspected GCA. The Göteborg criteria for temporal artery biopsy are polymyalgic symptoms and/or temporal-cranial symptoms + an increased sedimentation rate + age above 50 and no clinical signs of rheumatic disease other than GCA. Alternative criteria are: general symptoms of disease + an increased sedimentation rate. All the biopsies showed mononuclear inflammatory cell infiltration and giant cell reaction in the vessel wall. Thirty-four (79%) of the patients were women. Their mean age was 74.2 years (range: 56-88). As controls, 12 negative biopsies were included from age- and sex-matched patients who underwent temporal artery biopsy due to suspected GCA, but whose symptoms later were explained by other diseases, unrelated to GCA. Seven of the controls suffered from other forms of inflammatory disease (rheumatoid arthritis, n = 3; non-specified collagenosis, n = 1; non-specified polyarthritis, n = 1; pyelitis, n = 1; and chronic pyelonephritis, n = 1). The biopsies were fixed in a formaldehyde solution, cut into 1-3 mm thick slices, dehydrated and embedded in paraffin. Cross-sections were stained according to van Gieson, and with a combination of van Gieson and elastin staining. Other 5 µm thick sections were stained immunocytochemically. After microwave treatment, the sections were blocked with hydrogen peroxide to prevent endogenous peroxidase activity, and with normal rabbit serum to prevent non-specific staining. The sections were stained with antibodies to two different sites on the estrogen receptor protein: 1) a monoclonal mouse IgG1 antibody which reacts with the N-terminal domain (A/B region) of the re-
Estrogen receptors in giant cell arteritis / V. Petursdottir et al.

Western blot study
For the Western blot study, 4 inflamed GCA arteries and 4 non-inflamed, non-GCA controls were included. The biopsies were frozen in liquid nitrogen and kept at -80°C until analysed. Tissue from an immunocytochemically ER-positive breast carcinoma was used as a positive control. The frozen material was homogenized in 10 mM Tris buffer containing 1.5 mM EDTA, 50 mM NaF, 1 mM Na-orthovanadate, 1% triton X, 10 µg/ml leupeptin, 1 µg/ml aprotinin, 12 mM monothioglycerol and 4 mM pefa block. Cell homogenates were sonicated for 2 x 5 sec and were then centrifuged at 16,000 x g for 10 min at +4°C. From the supernatant, 30 mg protein was separated by electrophoresis on NuPAGE 4-1532-1509 (12)). The PCR analysis was performed as above. The amplified fragments were separated by electrophoresis in 1.5% agarose gel and visualized with ethidium bromide. cDNA from uterus was used as a positive control (Clontech, CA, USA).

Table I. The percentage of the biopsies in which the specified types of cell displayed immunoreactivity.

<table>
<thead>
<tr>
<th></th>
<th>ER-1D5</th>
<th>ER-LH2</th>
</tr>
</thead>
<tbody>
<tr>
<td>GCA patients</td>
<td>100%</td>
<td>100%</td>
</tr>
<tr>
<td>Smooth muscle</td>
<td>100%</td>
<td>100%</td>
</tr>
<tr>
<td>Giant cells</td>
<td>100%</td>
<td>100%</td>
</tr>
<tr>
<td>Angulated cells</td>
<td>100%</td>
<td>100%</td>
</tr>
<tr>
<td>Cell clusters</td>
<td>100%</td>
<td>92%</td>
</tr>
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Results

Immunocytochemistry
An ER-1D5-positive reaction was detected in all the inflamed temporal arteries from cases of GCA. The smooth muscle cells in the media displayed a positively-stained granular cytoplasm, with the most distinct positivity around the nuclei (Fig. 1a). Obvious nuclear staining was not identified. Smooth muscle cells in the thickened intima also displayed distinct cytoplasmic positivity. The data are shown in Table I.

Fig. 1. (a) Temporal artery media from a patient with GCA. Red immunopositivity to estrogen receptor α in the smooth muscle cells. (b) Temporal artery media from a control patient without GCA. (DAKO-ER-1D5 + haematoxylin. Bar: 15 mm)
mononuclear cell infiltration of varying degrees was seen around these granulomatous cell clusters, as well as in other parts of the arterial wall displaying scattered mononuclear, macrophage-like, ER-positive cells. Some of the lymphocytes also displayed ER positivity, but it was not as distinct as that in the smooth muscle cells, the angulated cells and the giant cells.

The majority of the biopsies stained positively for the other type of the ER antibody (LH2) (see Table I). The staining was paler, but the positivity was detected in the same cells and with the same pattern as that described above for ER-1D5. The macrophage staining was widely and strongly positive in the inflamed segments. Some of the angulated cells adherent to the LEI, most of the giant cells and multiple mononuclear cells in the diffusely distributed inflammatory infiltrate were CD-68 positive (Fig. 2c).

The angulated cells, the giant cells and many of the other inflammatory cells were positive to HLA-DR (Fig. 2b). Light-microscopically identified smooth muscle cells were α-smooth muscle actin-positive.

The majority of the 12 biopsies of non-inflamed arteries from the age- and sex-matched controls who did not receive a diagnosis of GCA displayed cytoplasmic positivity in smooth muscle cells to both ER antibodies (Fig. 1b, Table I).

A strong nuclear immunoreactivity to both ER antibodies was noted in the breast carcinoma control tissue. Some of these cells also displayed a weaker cytoplasmic staining (Fig. 4).

All the negative controls were totally free from immunoreactivity (Fig. 2d).
Discussion

Studies of the influence of estrogen on the vessel wall have focused predominantly on its relationship to atherosclerosis and more recently to chronic rejection in transplants. The low incidence of vascular disease in premenopausal women is well known (13, 14). Estrogen influences the serum lipid levels and the lipid metabolism (15, 16) and is thus indirectly atheroprotective. Recent studies have identified ER in human vascular smooth muscle cells (VSMC) in culture, indicating that estrogen may also have direct effects on vessel-wall metabolism (8, 17). In several studies, an inhibitory effect on VSMC growth has been reported and this might further contribute to the atheroprotective effect of estrogen (18, 19). Conversely, Keyes et al. demonstrated that estrogen, together with platelet-derived growth factor (PDGF) and protein kinase C, stimulates the growth of cultured VSMC from pregnant guinea pigs (20). Estrogen might thus play a role in preserving the normal vessel wall by stimulating as well as inhibiting the growth of VSMC, probably depending on the concentration and/or the presence of other stimulatory or inhibitory factors.

Our group has previously shown that non-inflamed temporal arterial segments from patients with GCA are atrophic, asymmetrical and calcified, compared with arteries from sex- and age-matched patients without GCA (3). A morphological study of giant cell arteritis supports these results (21). Our observations indicate that the inflammation starts as a foreign-body giant cell reaction directed at such calcifications. It thus seems that age-related arterial atrophy and calcification is a prerequisite for GCA. Possibly this degeneration is related to estrogen metabolism and to a reduced protection of the vessel wall. However, in the present study ER were detected immunocytochemically in the cytoplasm of smooth muscle cells in GCA patients as well as in controls, and in women as well as in men, which may suggest the involvement of estrogen not only in GCA but also in normal vascular aging. Somatic mutations may increase progressively with age, causing an accumulation of aberrant proteins within cells and

Western blot study

There was a close correlation between the bands in the GCA material, the non-GCA control arteries and those in the tumour controls (Fig. 5). The major band in all three groups corresponded to a molecular weight of approximately 64 kDa, as expected. Moreover, a band corresponding to 54 kDa was constantly present. Two further bands, corresponding to a molecular weight of approximately 40 and 45 kDa were found in the breast carcinoma.

RT-PCR

The inflamed and the non-inflamed biopsies, as well as the uterus control, displayed a predominant band corresponding to approximately 670 bp, as expected. A weak band was also seen, corresponding to approximately 440 bp (Fig. 6).
tissues (22). A recent epidemiologic study indicates that former pregnancies may be a protective factor against GCA (23), which supports the contention that estrogen could be pathogenetically involved. Further studies are needed to establish whether GCA vessels display structural or quantitative changes in the ER mRNA or in the receptor protein compared with controls.

The strongest ER immunoreactivity found in the present study was in angulated cells located on the outside of the LEI and in the giant cells. The same types of cell also displayed immunopositivity to HLA-DR and macrophage markers. A somewhat weaker immunoreactivity was seen in scattered macrophages within the diffuse mononuclear infiltrate. The immunocytochemical staining pattern with the cytoplasmic upgrading of ER in HLA-DR-positive macrophages and giant cells indicates that estrogen metabolism might be involved in the inflammatory process in GCA.

Previous studies have repeatedly indicated that sex hormones can influence the immune system. Women have a higher immunoglobulin concentration in the blood, increased resistance to a variety of infections and a decreased graft rejection time (10, 11). Estrogen has been shown to inhibit T suppressor cells, as well as are aberrant proteins or isofoms (35, 36). In addition to the major 64 kDa band, which corresponds to the size of the wild type ER, we found a band corresponding to 54 kDa in all of our Western blots, which might indicate the presence of at least one isoform [cf. (36)]. However, although we recognized two bands in the RT-PCR lanes, it remains unclear whether the shorter DNA strand might correspond to the same isoform.

ER have previously been immunocytochemically visualized in macrophages, osteoclasts (29, 37, 38) and in giant cells in giant cell lesions of the jaws (39). On the other hand, ER have not previously been described in Langhans giant cells or in foreign-body giant cells in granulomatous inflammation and never in the inflamed artery in GCA. Further studies of the molecular biology of ERα and the newly detected ERβ in GCA are now in progress.

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Estrogen receptors in giant cell arteritis / V. Petursdottir et al.


