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CONCISE REPORT

Smoking induces transcription of the heat shock protein system in the joints

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ABSTRACT

Objectives Smoking increases the risk of developing rheumatoid arthritis (RA) and worsens the course of the disease. In the current study we analysed whether smoking can affect gene expression directly in the joints.

Methods Synovial fibroblasts were incubated with 5% cigarette smoke extract and changes in gene expression were detected using whole genome microarrays and verified with real-time PCR. Synovial tissues were obtained from smoking and non-smoking patients with RA undergoing joint replacement surgery and from mice exposed to cigarette smoke or ambient air in a whole body exposure chamber for 3 weeks.

Results Microarray and real-time PCR analysis showed a significant upregulation of the heat shock proteins DnaJA4, DnaJB4, DnaJC6, HspB8 and Hsp70 after stimulation of synovial fibroblasts with 5% cigarette smoke extract. Similarly, in synovial tissues of smokers with RA the expression of DnaJB4, DnaJC6, HspB8 and Hsp70 was significantly higher compared with non-smokers with RA. Upregulation of DnaJB4 and DnaJC6 in joints by smoking was also confirmed in mice exposed to cigarette smoke.

Conclusions Our data clearly show that smoking can change gene expression in the joints, which can lead to the activation of signalling pathways that promote development of autoimmunity and chronic joint inflammation.

INTRODUCTION

Smokers have a higher risk of developing autoantibody-positive rheumatoid arthritis (RA) and smoking patients with RA show a more severe disease course.[1 2] The mechanisms by which smoking influences the development and the course of RA are not clear. A strong gene-environment association for smoking was found in patients with RA, in which only smokers who are carriers of the human leukocyte antigen (HLA) risk alleles for RA are at risk to develop anticitrullinated protein antibodies positive RA.[3] Since the formation of anticitrullinated protein antibodies can precede the disease onset for years and smoking was shown to induce citrullination of proteins in the lung, it was speculated that the disease starts in the lung and is redirected to the joints by cross-reactive mechanisms.[4 5]

Based on the variable systemic effects of smoking and the fact that the synovial fluid is a passive filtrate of blood plasma, it is reasonable to assume that smoke derivatives can enter the joints. To find effects of smoking in the joints, we used an in vitro model of smoke exposure, an in vivo mouse model and synovial tissues of smokers and non-smokers with RA.

METHODS

Synovial fibroblasts

Synovial fibroblasts were isolated as described in the online supplementary methods.

Synovial tissues

Synovial tissues from smoking and non-smoking patients with RA were obtained during joint replacement procedures at the Schulthess Clinic Zurich, Switzerland. Patients with RA fulfilled the 1987 American College of Rheumatology criteria for the classification of RA and signed a consent form.[6]

Mouse experiments

Male C57BL/6 mice were exposed to smoke from research cigarettes (University of Kentucky 1R4F) 6 h a day, 5 days per week in a whole-body smoking chamber (Teague TE-10). Cigarettes were smoked at a rate of a 2 s, 35 mL puff each minute and smoke particulate concentration was kept at 25.2 mg/m^3. Control mice were handled identically but exposed to filtered ambient air. After 3 weeks mice were euthanised and ankle joints were dissected. The results from four independent experiments are shown.

Cigarette smoke extract

Cigarette smoke extract (CSE) was prepared based on the method used by Vassallo *et al*.[7] Smoke was pulled from a cigarette into a syringe and via a three-way stopcock through a tube containing 10 mL prewarmed cell culture medium (eight pulls per cigarette). The cigarettes used contain 10 mg tar, 0.8 mg nicotine and 10 mg carbon monoxide (Marlboro Red). The resulting CSE (100%) was filtered through 0.2 μm filters and further diluted to 5% CSE.

Immunohistochemistry

Mouse slides were stained for glycophorin A using a validated standard protocol on a Ventana automat (Ventana Medical System, Tucson, Arizona, USA) with mouse antihuman glycophorin A antibodies (CD235a, clone JC159 1:100) (Dako, Baar, Switzerland). Detection was performed with respective secondary antibodies and Ultraview Amp kits (Ventana). Positive cells were counted in three random fields of view at a magnification of 200×. On average 1500 cells per mouse were counted.

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RNA extraction

For extraction of RNA from tissues, snap frozen tissue was grinded and processed with TRIzol (Invitrogen, Carlsbad, California, USA) according to the manufacturer's protocol. RNA from cells was isolated with the RNeasy Mini Kit (Qiagen, Basel, Switzerland) including DNase digestion.

Microarrays

Total RNA from CSE incubated RA synovial fibroblasts (RASF) and control RASF (n=1 each) was isolated after 24 h as described above. Labelling, hybridisation and analysis of the samples was done in the Functional Genomic Center Zurich as previously described.[8] GeneChip Human Genome U133 Plus Arrays (Affymetrix) were used. Raw data were processed as described in the online supplementary methods and analysed with the Database for Annotation, Visualization and Integrated Discovery (DAVID V.6.7).[9]

Real time PCR

Reverse transcription and calculation of relative expression levels using the comparative threshold cycle method was done as described in the online supplementary methods.

Statistics

For paired samples Wilcoxon signed rank test and for unpaired samples Mann-Whitney test were applied and p values <0.05 were considered statistically significant (GraphPad Prism Software V.5.0). Values are presented as mean±SEM.

RESULTS

CSE induces expression of chaperones in RASF

To screen for pathways that are activated by constituents of cigarette smoke in synovial fibroblasts, RASF were stimulated with 5% CSE and genome-wide changes in gene expression were measured. Functional annotation clustering of the top 100 reads after 5% CSE (see online supplementary table S1) found significant clusters

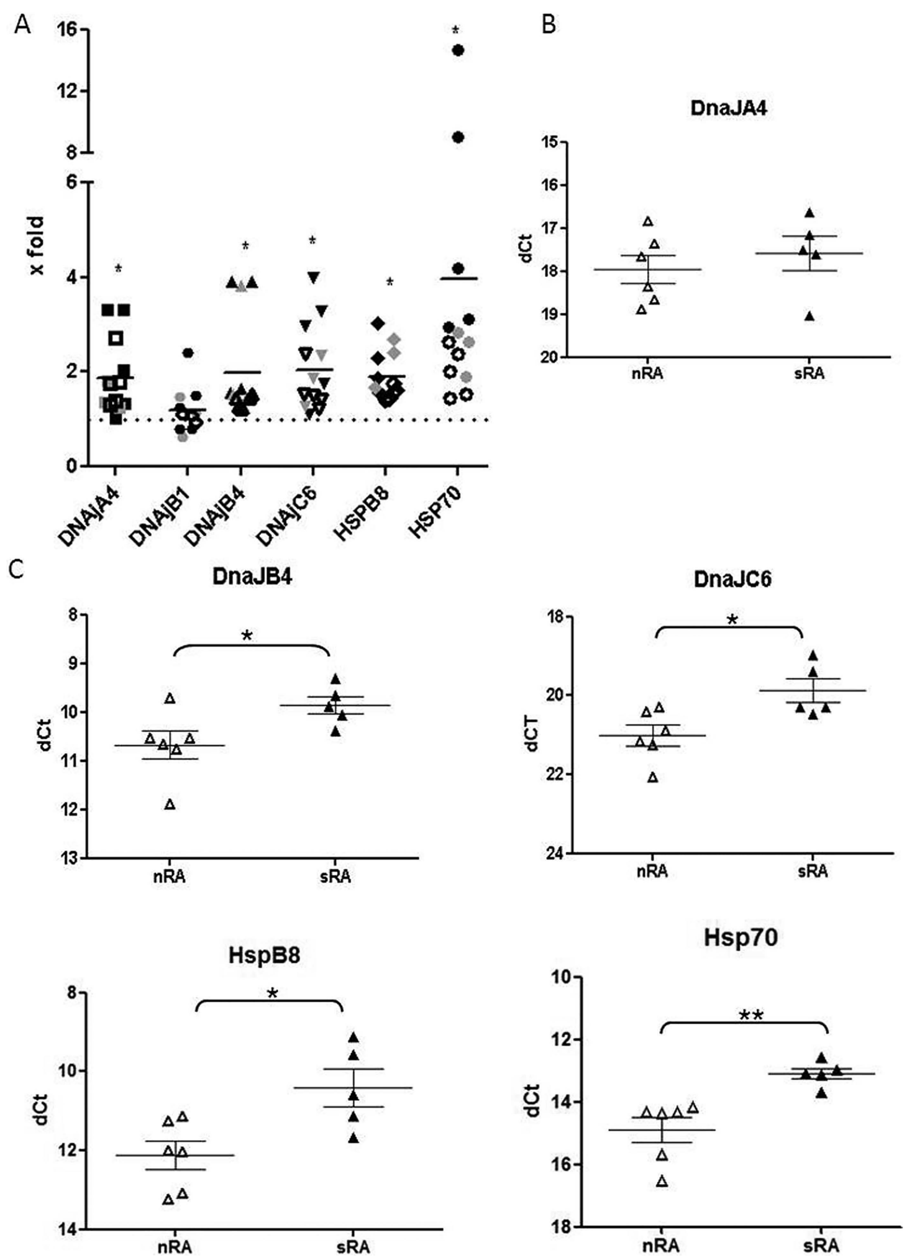

Figure 1 Cigarette smoke changes the expression of heat shock proteins in vitro and in vivo. (A) Stimulation of rheumatoid arthritis (RA) synovial fibroblasts from non-smokers (black symbols) and smokers (grey symbols) and of synovial fibroblasts from non-smokers with osteoarthritis (empty symbols) with 5% cigarette smoke extract significantly increased the expression of heat shock proteins as compared with unstimulated cells (basal level set to 1 as indicated by the dotted line; *p<0.01 determined by Wilcoxon signed rank test). In synovial tissues of patients with RA there was no difference in the levels of DnaJA4 between smokers (sRA) and non-smokers (nRA) (B), but levels of DnaJB4, DnaJC6, HspB8 and Hsp70 were significantly higher expressed in synovial tissues of smoking patients with RA (C). dCt, difference of cycle of threshold between the endogenous control 18 s and the target gene; *p<0.05, **p<0.01 as determined by Mann-Whitney test.

for the gene ontology term 'heat-shock protein binding' (p=2.5E-6) and the protein information resource keyword 'chaperone' (p=5.5E-5). This group consisted of heat shock protein (HSP) 22 kDa protein 8 (HspB8), Hsp70 and five members of the HSP40 family, namely DnaJA4, DnaJB1, DnaJB4, DnaJB9 and DnaJC6. Upregulation of all of these chaperones except for DnaJB1 and DnaJB9 by 5% CSE was confirmed in RASF from smokers as well as non-smokers and from synovial fibroblasts derived from patients with osteoarthritis (figure 1A).

Elevated levels of chaperones in joints of smokers

We measured the expression of these chaperones in human synovial tissues from smoking and non-smoking patients with RA (for characteristics of patients see online supplementary table S2). There was no difference between the expression levels of DnaJA4 between smokers and non-smokers (figure 1B). In contrast transcript levels of DnaJB4, DnaJC6, HspB8 and Hsp70 were significantly higher in synovial tissues of smokers than of non-smokers (figure 1C). Smokers expressed two times more of the measured DnaJ transcripts (1.8-fold DnaJB4 and 2.2-fold DnaJC6) and more than three times more of the HSPs (3.2-fold HspB8 and 3.5-fold Hsp70).

DnaJ transcripts are induced in joints of smoke-exposed mice

To further test the hypothesis that smoking can change gene expression in joints, a mouse model of smoke exposure was used. Since it is well established that human smokers develop polycythaemia with elevated haematocrits, erythropoiesis was analysed as a marker of systemic effects of smoking.[10] Staining for the erythroid marker glycophorin A in bone marrow showed a significant increase in erythroid precursor cells, proving that similar systemic changes as in human smokers were induced in mice (figure 2A).

While no infiltration of the joints by macrophages or lymphocytes was elicited by smoking, smoke exposure induced a 2.2-fold increase of DnaJB4 and a 2.7-fold increase of DnaJC6 in ankle joints (figure 2B). However, no change in the levels of HspB8 or Hsp70 was associated with smoke exposure (figure 2C).

CONCLUSIONS

Our work clearly shows that smoking alters gene expression in joints and thus local changes in the joints induced by smoking can confer the risk of developing RA and influence the severity of the disease course.

Since cigarette smoke contains thousands of different substances, it is difficult to speculate which of the compounds reach the synovial fluid and activate joint resident cells. A previous study showed that the aryl hydrocarbon receptor which can be activated by polycyclic aromatic hydrocarbons included in smoke is expressed in synovial tissues.[11] This receptor was activated in synovial tissues of smokers as shown by elevated levels of CYP1A1 and aryl-hydrocarbon receptor repressor (AHRR). Moreover, the α7 subunit of the nicotinic acetylcholine receptor was shown to be expressed in the synovium.[12] In vitro, nicotine

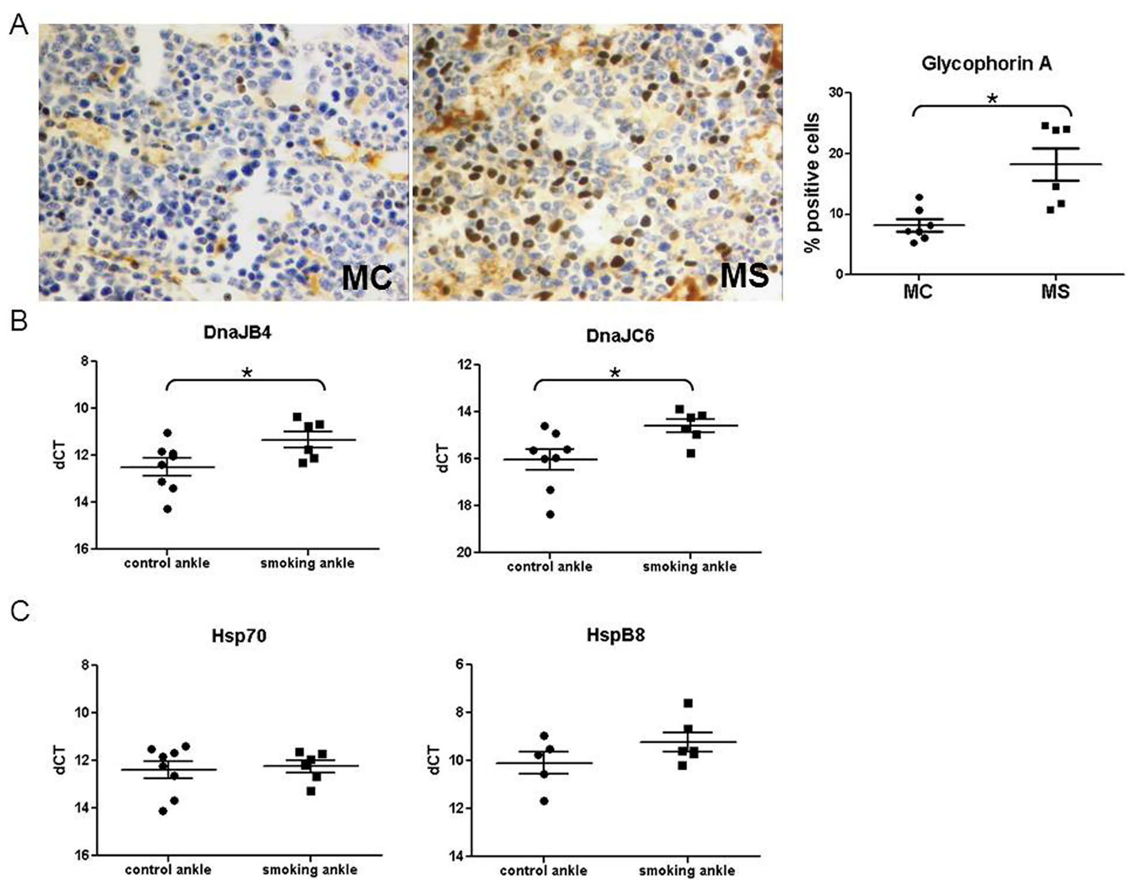

Figure 2 Effects of exposure to cigarette smoke in mice. (A) The erythroid marker glycophorin A was increased in mice exposed to cigarette smoke (MS) compared with mice exposed to ambient air (MC). Representative pictures are shown; glycophorin A is stained in brown, nuclei are counterstained in blue; magnification ×200. The expression of DnaJB4 and DnaJC6 (B), but not of Hsp70 and HspB8 (C) was significantly increased in ankle joints of smoke exposed mice (smoking ankle) compared with control mice (control ankle). dCt, difference of cycle of threshold between the endogenous control 18 s and the target gene; *p<0.05 as determined by Mann-Whitney test.

reduced the expression of proinflammatory cytokines by synovial fibroblasts and is thus considered to be an inhibitor of inflammation.[12]

It is widely acknowledged that the immunogenicity of a peptide is increased when it is bound to Hsp70.[13] This mechanism can be used to induce an immune response against tumour cells and to increase the efficiency of vaccines. Furthermore, high levels of Hsp70 were implicated in break of tolerance against self-peptides and are suspected to induce autoimmunity.[14] Elevated levels of Hsp70 were also detected in brains and intervertebral discs of rats exposed to cigarette smoke.[15 16] Therefore, increased levels of Hsp70 in tissues of smokers might promote the formation of autoantibodies against tissue structures and, depending on the genetic background, induce autoimmunity at different sites.

Based on similarities of the human and bacterial DnaJ motifs and the fact that DnaJ from *Escherichia coli* contains the 'shared epitope' motif, cross-reaction of activated T cells with self DnaJ was proposed to promote autoimmunity in RA.[17] Therefore a synthetic DnaJ peptide (DnaJP1) was developed to restore tolerance and improve clinical symptoms of RA.[18] This treatment approach has already been successfully applied in various animal models of autoimmunity.[19]

Members of the HSP family were also described to activate the innate immune system via toll-like receptors (TLRs). In particular, Hsp70 was found to activate TLR2 and TLR4, and HspB8 to activate TLR4.[20 21] TLR stimulation of synovial fibroblasts is a major contributor to the pathogenesis of RA and increases the production of inflammatory cytokines and of matrix-destructive molecules. However, there is reasonable concern that at least some of the results showing TLR activation by HSPs stem from contaminants in the HSP preparations.[22]

Hsp70 and HspB8 were not upregulated in mice after smoke exposure. This discrepancy might stem from differences in the HSP response between humans and mice. In humans as well as in mice the genetic background plays a substantial role in the response to smoke. In humans, expression of the RA risk alleles HLA-DRB1 is strongly connected to the risk of smokers developing RA.[3] In mice, smoke-induced lung inflammation and signalling pathways differ between strains.[23] Therefore, it might be that the genetic background also influences the quality and quantity of the HSP response.

In summary, our data clearly show that by smoking, local changes in gene expression can be induced in joints, which can lead to the activation of signalling pathways that promote autoimmunity and chronic joint inflammation.

Contributors All authors included on this paper fulfil the criteria of authorship and that there is no one else who fulfils the criteria but has not been included as an author.

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Competing interests None.

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Ethics approval Swiss Ethical Commission.

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