Smoking and Thyroid-Associated Ophthalmopathy: A Novel Explanation of the Biological Link

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Introduction: Cigarette smoking is the strongest modifiable risk factor for developing thyroid-associated ophthalmopathy (TAO), and the severity of TAO is related to the current number of cigarettes smoked per day. We aimed to establish the effects of cigarette smoke extract (CSE) on an *in vitro* model of TAO.

Methods: Orbital tissue was taken during surgery from 10 patients with TAO and nine control subjects. Orbital fibroblasts were cultured and exposed to CSE, and intercellular adhesion molecule 1 (ICAM1) expression was measured by flow cytometry. Glycosaminoglycan production was measured by hyaluronic acid ELISA. Orbital fibroblasts were grown in adipogenic media with or without CSE and/or IL-1, and the degree of adipogenesis was quantified.

Results: Fibroblasts from patients with TAO and controls showed similar responses. ICAM1 expression was not affected by CSE. Hy-aluronic acid production was stimulated by CSE in a dose-dependent manner (correlation coefficient, 0.978; $P = 0.022$), with 5% CSE causing an increase of 44% ($P = 0.001$). CSE increased adipogenesis in a dose-related manner, as did IL-1. The effects of CSE and IL-1 on adipogenesis were synergistic, with the degree of adipogenesis in the well containing both 5% CSE and 0.1 ng/ml IL-1 being double the magnitude of the sum of the values obtained from either stimulus alone ($P < 0.001$). Addition of an anti-IL-1 antibody to the well containing both 5% CSE and 0.1 ng/ml IL-1 reduced the degree of adipogenesis by 82% ($P < 0.001$).

Conclusion: These findings may help explain how cigarette smoking has a detrimental effect in TAO and suggests that IL-1 may be an attractive therapeutic target in TAO. (*J Clin Endocrinol Metab* **92: 59–64, 2007**)

T HYROID-ASSOCIATED OPHTHALMOPATHY (TAO) is an autoimmune inflammatory condition that affects 25–50% of patients with Graves' disease (1). TAO is also known as Graves' ophthalmopathy and thyroid eye disease. The key pathological features of TAO within the orbit are inflammation, excess production of glycosaminglycans (GAG), and adipogenesis, and these processes are thought to be driven at least in part by the local release of inflammatory cytokines (1, 2). The strongest modifiable risk factor for developing TAO is smoking.

Cigarette smoking affects the incidence, the severity, and the response to treatment in TAO and appears to do so in a dose-related and temporal manner. Smokers with Graves' disease are approximately five times more likely to develop TAO than nonsmokers with Graves' disease (3–7). Evidence for a dose-response relationship between smoking and the severity of TAO includes the severity of TAO being related to the current number of cigarettes smoked per day (3, 4, 6, 8) and the percentage of heavy smokers being higher in patients with more severe ophthalmopathy (5). It appears that current, but not lifetime, tobacco consumption constitutes a risk for the incidence of proptosis and diplopia in patients with TAO and that this risk increases with the number of cigarettes smoked per day (9). Temporality is also suggested by the few prospective studies, as is reversibility because former smokers have a lower risk of developing TAO than current smokers, even with a comparable lifetime tobacco consumption (7). Smoking also influences the course of TAO during treatment in a dose-dependent manner. The response to treatment is delayed and considerably poorer in smokers (10). Furthermore, smoking increases the risk of progression of ophthalmopathy after radioiodine therapy (11), and stopping smoking currently represents the only form of TAO prevention (12).

Despite the established association between smoking and TAO, the mechanism by which smoking affects TAO is not known. It has been proposed that the formation of superoxide radicals and tissue hypoxia may be involved. Superoxide radicals can induce orbital fibroblasts (OFs) from patients with TAO to proliferate in a dose-dependent manner (13), and cigarette smoke either contains or can generate a variety of oxidants and free radicals (14, 15). Tissue hypoxia (5% CO_2 and 95% N_2) can also stimulate orbital fibroblasts, both to proliferate and to synthesize GAG (16). However the lack of any reported link between high-altitude living and TAO and the relatively modest degree of tissue hypoxia in smokers without severe lung disease might argue against hypoxia being a major direct influence in TAO *in vivo*. Cultured orbital fibroblasts have been shown to increase their expression of human leukocyte antigen (HLA-DR) in response to nicotine and tar but only when in the presence of interferon-γ, suggesting a possible mechanism by which smoking can alter orbital immune responses in TAO (17). Thyroid hormones and TSH receptor autoantibodies are not

affected by smoking, but serum TSH levels have been found to be slightly reduced and smokers have a higher frequency of goiter and increased serum thyroglobulin levels, especially in iodine-deficient areas (18).

We aimed to examine the effects of cigarette smoke extract (CSE) on an *in vitro* model of TAO. The three endpoints assessed reflect the three key pathological processes in TAO, namely inflammation, GAG production, and adipogenesis (19).

Subjects and Methods

In vitro model of TAO

The *in vitro* model of TAO consists of primary cultures of OFs from patients with TAO, as described previously (19, 20). Orbital fat biopsies were taken at surgery and transported to the laboratory in normal saline at room temperature. The biopsies were diced using a scalpel blade and placed in RPMI tissue culture medium containing 9% fetal bovine serum, 0.02 M HEPES, 1.8 mg/liter fungizone, 35 U/ml penicillin, and 35 μg/ml streptomycin in a 25-cm^2 flask and grown at 37 C in 5% CO_2 and 21% O_2 (additives from Biosciences Ltd, Invitrogen, Dun Laoghaire, Ireland). Once OFs were adherent, the flask was washed with culture medium and OFs grown to confluence with medium replaced every 3–4 d.

Subjects

Subjects consisted of patients with TAO, and a control group of patients without TAO were also studied (Table 1). TAO was diagnosed on clinical grounds based on the presence of typical clinical features in the context of autoimmune thyroid disease. Control subjects were patients without a personal or family history of thyroid disease or TAO and without clinical evidence of TAO, who were attending for orbital surgery. The study was approved by the institutional ethics committees, and all study participants gave written informed consent.

CSE

The CSE generation system was based upon a validated pump system (21). Four cigarettes were smoked through 30 ml culture medium. Each cigarette was smoked for 10 puffs, each puff being 35 ml smoke, every 30 sec, which burnt approximately 75% of the cigarette, and the volume of smoke generated per cigarette was 350 ml. Each milliliter of CSE contains

0.133 (4/30) cigarette's worth of smoke-derived constituents. The resultant CSE was sterilized by filtering through a 0.2-μm filter, pH adjusted to 7.4, and then stored at -20 C. The cigarettes used were Marlboro Reds, Class A, rated as tar 10 mg, nicotine 0.8 mg, and carbon monoxide 10 mg.

Bernhard's validated volumetric calculations (21) are based on the assumptions that a human generates 350 ml smoke with each cigarette and has a blood volume of 6 liters. If that person smokes 20 cigarettes per day, then each 300 ml (6000/20) of blood contains the equivalent of one cigarette. Therefore, each milliliter of blood contains 0.0033 (1/300) cigarette's worth of smoke-derived constituents. By this calculation, CSE can be considered to contain 40 times (0.133/0.0033) the amount of smoke-derived constituents than would be expected to be contained in the blood of a smoker who smokes 20 cigarettes per day. A 2.5% (1/40) solution of CSE would equate to 20 cigarettes per day. We used between 1.25% CSE and 12.5% CSE, which equates to between 10 and 100 cigarettes per day, respectively.

Intercellular adhesion molecule 1 (ICAM1) measurements

As a marker of the inflammatory response, the expression of cell-surface ICAM1 by OFs was examined by culturing OFs to confluence in 12-well plates and then stimulating for 24 h with either CSE or selected cytokines and comparing these to unstimulated conditions on each plate. Flow cytometry was used to measure the relative expression of ICAM1 (19). OFs were trypsinized, centrifuged, and resuspended in 200 μl culture medium. OFs were then added to 5 μl isotype IgG control phycoerythrin mouse antibody (Becton Dickinson, No. 345816; Oxford, UK) or 5 μl of anti-ICAM1 phycoerythrin antibody (Becton Dickinson, No. 555511), vortexed and incubated in darkness at 4 C for 30 min. OFs were washed with phosphate-buffered azide and resuspended in 1% paraformaldehyde. The OFs were analyzed using a Becton Dickinson FAScan cytometer and Cellquest analysis software with data expressed as median fluorescent intensity of each experimental condition, in arbitrary units. Interassay and intraassay coefficients of variation were 2.7 and 2.2%, respectively. Cytokines TNFα, IL-1, interferon-γ (IFNγ), TGFβ, and IL-10 were purchased from R&D Systems (Abingdon, UK). The anti-IL-1 antibody used was anakinra (Amgen, Cambridge, UK; 5 μg/ml, a level found in serum after therapeutic administration) (19, 22).

GAG measurements

OFs were grown to confluence in 12-well plates and then stimulated with CSE for 48 h. The hyaluronic acid (HA) levels in the supernatants

TABLE 1. Clinical details of TAO patients and control subjects

Sex	Age (yr)	Duration of TAO (yr)	Clinical activity score	Cigarette smoker	Previous orbital radiotherapy	Previous or current steroids for TAO	Surgical procedure
TAO							
Female	65	1.5	1	No	Yes	Yes	Lid recession
Female	47	16	1	Yes	No	Yes	Lid recession
Female	53	0.8	9	Yes	No	Yes	Emergency orbital decompression
Female	51	0.6	2	Yes	No	No	Lid recession due to exposure keratopathy
Female	44	5	0	No	No	Yes	Orbital decompression
Male	42	4.5	0	No	Yes	Yes	Orbital decompression
Female	46	5	0	Yes	No	Yes	Lid recession
Female	66	1	5	Yes	No	Yes	Emergency orbital decompression
Female	44	10	2	Yes	No	Yes	Lid recession
Female	44	11	1	Yes	Yes	Yes	Blepharoplasty
Controls							
Male	53	NA	0	No	No	NA	Ptosis repair
Female	48	NA	0	No	No	NA	Ptosis repair
Female	21	NA	0	No	No	NA	Congenital ptosis repair
Female	77	NA	0	No	No	NA	Ptosis repair
Female	46	NA	0	No	No	NA	Blepharoplasty
Female	63	NA	0	No	No	NA	Sphenoid meningioma resection
Male	33	NA	0	No	No	NA	Dermis fat graft (previous enucleation)
Female	57	NA	0	No	No	NA	Blepharoplasty
Female	54	NA	0	No	No	NA	Blepharoplasty

Clinical activity score refers to the Mourits clinical activity score, with 10 representing most active disease and 0 least active disease. NA, Not applicable.

from these experiments were measured using an ELISA kit (K-1200; Echelon Biosciences Inc., Salt Lake City, UT) as per the manufacturer's instructions (19). Samples were diluted 1:10 before analysis, and the average of duplicate measurements was taken. Interassay and intraassay coefficients of variation were 10.8 and 6.6%, respectively.

Adipogenesis

OFs were exposed to a differentiation protocol to encourage adipogenesis according to published methods (19, 23–27). OFs were grown to confluence in multiwell plates and exposed to differentiation media for 4 d (RPMI lacking fetal bovine serum and supplemented with 0.1 mM 3-isobutyl-1-methylxanthine, 1 μM dexamethasone, 1 μM rosiglitazone (Avandia; GlaxoSmithKline Ltd, Dublin, Ireland), 100 nM insulin (Actrapid; Novo Nordisk Ltd, Dublin, Ireland), 10 μg/ml transferrin, and 0.2 nM T$_3$ plus HEPES and antibiotics as detailed above. Medium was replaced after 4 and 8 d with propagation media (identical to differentiation media but lacking the 3-isobutyl-1-methylxanthine, dexamethasone, and rosiglitazone) and cells analyzed after 10 days. CSE and/or IL-1 and/or the anti-IL-1 antibody were added at the beginning of the differentiation protocol and continued for the 10 d, being replaced whenever medium was replaced. Cells were stained for lipid using oil red O, photographed, and assessed for degree of adipogenesis using three methods: 1) extraction of oil red O and measuring absorbance units at 492 nm (28), 2) a visual scoring system, and 3) computer analysis of digital photographs, as described in detail elsewhere (19). The values obtained using each technique were given equal weighting by normalizing to 100 using the IL-1 1.0-ng/ml well, and the mean of the three resulting measurements from each well was taken. In the 12-well plate experiments examining the effects of IL-1 and/or CSE, the values were adjusted by first subtracting the value from the control well to give a measure of change from baseline and then given equal weighting by normalizing to 100 using the well that displayed the maximum degree of adipogenesis on each plate. The mean of the three resulting measurements from each well were taken to give the final measure of adipogenesis (the composite adipogenesis score, in arbitrary units).

Statistics

The two-sided paired *t* test was used to test ICAM, GAG, and adipogenesis responses compared with the unstimulated control condition from each plate. Correlation of CSE concentration and HA production was done using Pearson product moment correlation. Statistical significance was taken as <0.05, and data were analyzed using Minitab 13 software.

Results

ICAM1 expression

Fibroblast ICAM1 expression was assessed in OFs from five patients with TAO and five control subjects by stimulating for 24 h with either CSE or selected cytokines. ICAM1 expression was not significantly increased by CSE up to 12.5% (100 cigarettes per day) (Fig. 1). In patients with TAO, the ICAM1 expression (average of the median fluorescent intensities, in arbitrary units) in basal conditions was 11.9 and after 24 h exposure to 12.5% CSE was 14.0 [95% confidence interval (CI), −0.62 to + 4.97; *P* = 0.1]. In control subjects, the median fluorescent intensity was 18.9 in basal conditions and after exposure to 12.5% CSE was 20.03 (95% CI, −2.86 to +5.16; *P* = 0.5).

The positive controls, the inflammatory cytokines TNFα, IL-1, and IFNγ (all at 0.1 ng/ml), each increased ICAM1 expression significantly (*P* values < 0.01). The negative controls, the antiinflammatory cytokines TGFβ (1.0 ng/ml) and IL-10 (10 ng/ml), did not significantly increase ICAM1 expression (*P* values 0.2–0.6) (Fig. 1).

GAG production

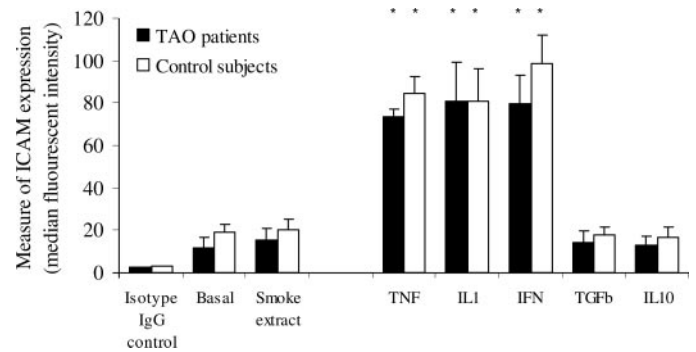In OFs from six patients with TAO and seven control subjects, CSE (0, 1.25, 2.5, and 5%, corresponding to 0, 10, 20,

FIG. 1. Effect of 24 h of exposure to CSE (12.5%, corresponding to 100 cigarettes/d) and TNFα (0.1 ng/ml), IL-1 (0.1 ng/ml), IFNγ (0.1 ng/ml), TGFβ (1.0 ng/ml), and IL-10 (10 ng/ml) on ICAM expression in OFs from patients with TAO and control subjects. Data are expressed as mean ± SEM. *, Statistically significant difference *vs.* basal condition (*P* < 0.05).

and 40 cigarettes/d) increased HA production in a dose-dependent manner (correlation coefficient r = 0.978 and *P* = 0.022 in TAO; r = 0.980 and *P* = 0.020 in control subjects).

Comparing HA concentrations in supernatants with that from OFs not exposed to CSE, the increases were statistically significant with 2.5 and 5% CSE, but not with 1.25% CSE (Fig. 2). Exposure of OFs from patients with TAO to 1.2%, 2.5, and 5% CSE for 48 h caused an increase in HA concentration in the OF supernatants of 18.4% (95% CI, −6.8 to 43.5; *P* = 0.12), 20.8% (95% CI, 9.8–31.7; *P* = 0.005), and 43.9% (95% CI, 26.0–61.8; *P* = 0.001), respectively. Exposure of OFs from control subjects to 1.25, 2.5, and 5% CSE for 48 h caused an increase in HA concentration in the OF supernatants of 3.0% (95% CI, −22.2 to 28.1; *P* = 0.78), 18.8% (95% CI, 4.1–33.5; *P* = 0.022), and 33.3% (95% CI, 2.6–64.1; *P* = 0.038), respectively (Fig. 2).

The tissue culture medium used for the GAG experiments and for the generation of CSE was identical and contained 9% fetal calf serum. This tissue culture medium and the CSE contained similar levels of HA (400–600 ng/ml).
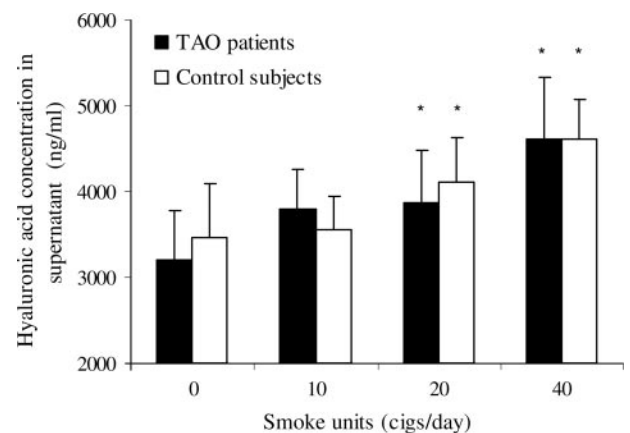

FIG. 2. The effect of 48 h of exposure to CSE (0, 1.25, 2.5, and 5%, corresponding to 0, 10, 20, and 40 cigarettes/d) on the HA concentration in supernatants of OFs from patients with TAO (n = 6) and from control subjects (n = 7) grown in 12-well plates. *, Statistically different concentration compared with that from fibroblasts not exposed to CSE (*P* < 0.05).

Adipogenesis

In keeping with previous reports, the ability of OFs to accumulate lipid generally decreased with increasing cell passage (19, 27), and so each plate contained cells from the same patient that had undergone the same number of passages, and each plate had its own control well. The mean passage number was three (range, one to six).

CSE (40 cigarettes/d) significantly increased adipogenesis compared with baseline conditions in OFs grown in six-well plates from both TAO patients (+34 in composite adipogenesis score; 95% CI, 4–64; $P = 0.031$; n = 9) and control subjects (+15; 95% CI, 7–23; $P = 0.003$; n = 9) (Fig. 3). The degree of adipogenesis seen with CSE (40 cigarettes/d) was not significantly different between TAO and control patients (80 *vs.* 62; 95% CI for difference, −15 to 53; $P = 0.25$).

To examine whether CSE has any additive or synergistic effects with IL-1, a cytokine known to promote adipogenesis in OFs (19), we cultured OFs from six patients with TAO in 12-well plates and examined the effects of CSE and IL-1, separately and in combination.

CSE increased adipogenesis in a dose-related manner, as did IL-1 (Fig. 4). Furthermore, the effects of CSE and IL-1 on adipogenesis were synergistic, with the degree of adipogenesis in the well containing both 40 cigarettes/d and 0.1 ng/ml IL-1 being double the magnitude of the sum of the values obtained from either stimulus alone [98.3 *vs.* 49.4 (37.3 for 0.1 ng/ml IL-1 + 12.1 for 40 cigarettes/d); 95% CI for difference, 34.1–63.7; $P < 0.001$] (Fig. 4). The addition of an anti-IL-1 antibody (5 μg/ml anakinra) to the well containing both 40 cigarettes/d and 0.1 ng/ml IL-1 reduced the degree of adipogenesis by 82.0% (98.3 *vs.* 17.5; 95% CI for difference, 62.7–99.0; $P < 0.001$) (Fig. 4).

Discussion

This study has shown that CSE increases GAG production and adipogenesis but not ICAM1 expression in an *in vitro* model of TAO. In addition, the adipogenic effects of CSE and IL-1 were synergistic.
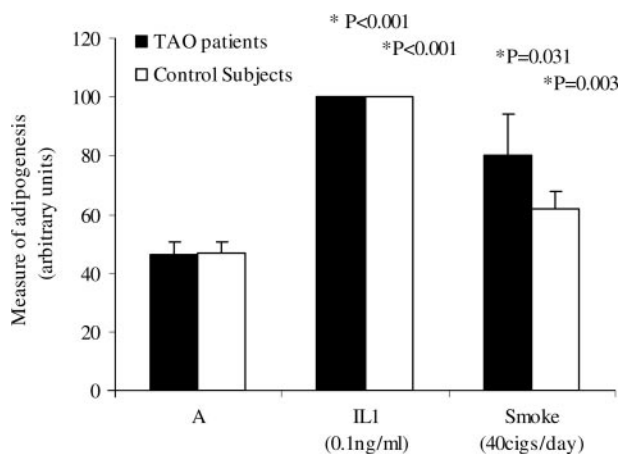

FIG. 3. The effect of exposure to IL-1 or CSE (5%, corresponding to 40 cigarettes/d), compared with the unstimulated well (A) that contained adipogenic medium but no cytokines or CSE, on adipogenesis in OFs grown in six-well plates from patients with TAO (n = 9) and control subjects (n = 9). Data are expressed as mean ± SEM. The P values shown are for the comparison of each condition with the corresponding unstimulated well (A).
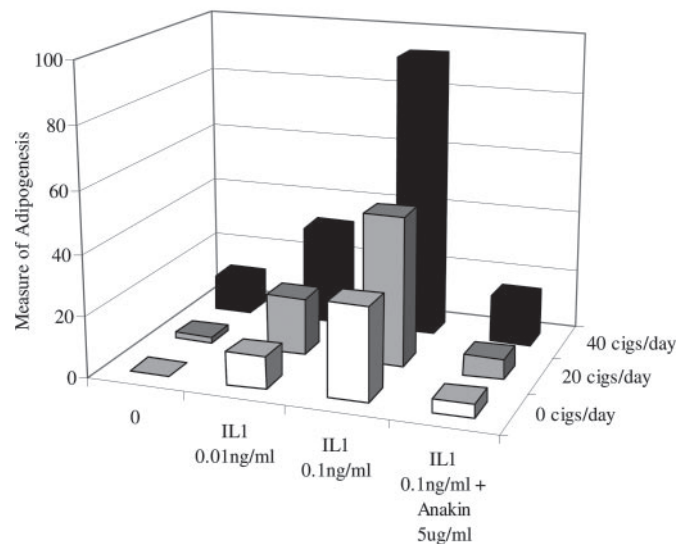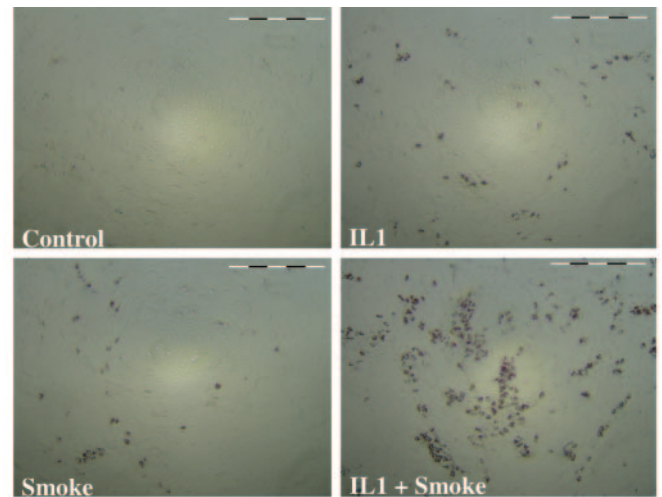

FIG. 4. *Top*, Digital photographs of OFs in culture showing the adipogenic effects of IL-1 (0.01 ng/ml) and CSE (5%, corresponding to 40 cigarettes/d), alone and in combination. The *dark spots* are intracellular lipid droplets stained with oil red O. The control well contains adipogenic medium but no cytokine or CSE. Total length of calibration bar is 1 mm, with each *black* or *white* section representing 200 μm. *Bottom*, Effect of CSE (0, 2.5, and 5%, corresponding to 0, 20, and 40 cigarettes/d) and IL-1, alone and in combination, on adipogenesis in OFs from six patients with TAO. Adipogenesis is measured by a composite score in arbitrary units that combines oil red O extraction, computer analysis of digital photographs, and visual assessment. Anakin represents the anti-IL-1 antibody anakinra (Amgen).

There are a number of *in vitro* approaches that have been used to study the effects of cigarette smoke. The commonly used techniques are to use single compounds, such as nicotine, or to use some form of CSE. Because cigarette smoke contains more than 4000 compounds, using a single-compound approach may fail to identify important effects if the incorrect compound is not chosen (29, 30). Using CSE is limited by the quality of the extract in that the extract may not reflect the full array of compounds found in the serum of smokers, nor may they be at the correct concentrations. For this current study, we decided to use CSE, because the active compound(s) in TAO have not yet been identified. Numer-

ous CSE-generating systems have been used, including syringe systems where the CSE is reported as a percentage (15, 31) and other methods, some of which attempt to relate CSE concentrations used *in vitro* to *in vivo* levels of certain compounds found in cigarette smoke (21, 29, 30, 32, 33). A system that had been extensively studied and validated was that by Bernhard *et al.* (21). This technique was therefore chosen, and a similar system to generate CSE was constructed. However, no *in vitro* model can exactly mimic the *in vivo* situation. Until the compound(s) in cigarette smoke that are responsible for the effects in TAO are identified, it is impossible to know how the concentrations of these compounds *in vitro* relate to the *in vivo* situation. In the absence of an accepted animal model, we used the human model of TAO, which involves culturing OFs taken from patients undergoing surgery for TAO (20, 34). This model has certain limitations (19) but nevertheless provides a useful tool to study the effects of cigarette smoke in TAO. In accordance with a previous study from our group, we did not find any obvious differences between the behavior of OFs from TAO patients and normal subjects, or between subgroups such as smokers and nonsmokers, or those exposed to previous steroid or radiotherapy treatment (19). However, this study was not designed to detect between-group differences. If OFs from TAO patients and healthy controls are not inherently different, it would suggest that it is the presence or absence of key cytokines in the orbit that influences whether TAO occurs, rather than the development of TAO being dependent upon interindividual differences in OF properties (19).

The finding that CSE increased both GAG production and adipogenesis presents a possible mechanism by which cigarette smoke worsens TAO; namely that chemicals present in cigarette smoke are carried in the circulation to the orbit, where they stimulate OFs, resulting in GAG production and adipogenesis and consequent tissue volume expansion. However, smoking itself, in the absence of autoimmune thyroid disease, is not associated with any clinically apparent orbital tissue expansion. It may be that the degree of GAG production and adipogenesis observed *in vitro* is not clinically significant *in vivo*. Against this is the fact that the magnitude of the *in vitro* effects of CSE (40 cigarettes/d) are approximately half that seen with IL-1 (0.1 ng.ml). Therefore, if IL-1 has significant effects *in vivo* in TAO, as is currently proposed (1, 35, 36), then the smoke effect is also likely to be clinically important. Another possibility is that cigarette smoke may have some additive or synergistic effect in combination with other pathogenic influences in the orbit, such as inflammatory cytokines or activated lymphocytes. Thus, only in the presence of an inflammatory response does the smoke effect become clinically important. This latter possibility is supported by the finding in this study of a synergistic effect of CSE and IL-1 on adipogenesis in OFs.

The observed synergy between IL-1 and cigarette smoke on adipogenesis may explain why smoking, in the absence of any local orbital inflammatory response, does not result in signs or symptoms similar to TAO. The presence of orbit inflammation and the consequent cytokine milieu may be required for the magnitude of the cigarette smoke effect to be clinically significant. The observed synergy between IL-1 and cigarette smoke may also explain the increased frequency of TAO in smokers because a large proportion of patients have TAO that is clinically undetectable but is present on radiological studies (37). If cigarette smoking increases the severity of disease above a threshold at which it becomes symptomatic, then the apparent incidence will be increased.

The finding that the IL-1 antagonist used in this study can block the IL-1 effect on adipogenesis and so abolish the synergy between CSE and IL-1 may have important therapeutic implications. This antibody is already in clinical use for the treatment of rheumatoid arthritis, and we have previously established that this agent can block IL-1-mediated increases in ICAM1 and GAG production in OFs *in vitro* (19). Smokers are more likely to experience severe disease than nonsmokers and are less likely to respond to currently available medical treatments (10). Therefore, the interruption of synergy between the effects of smoke and cytokines in this *in vitro* model of TAO makes this therapeutic strategy particularly attractive for those who smoke, and clinical trials are now needed to test this hypothesis.

In contrast to inflammatory cytokines such as TNFα and IL-1, CSE had no effect on ICAM1 expression. This lack of effect of CSE on ICAM1 expression in OFs suggests that CSE is not having an effect on TAO via this aspect of the innate immune system and CSE is not acting in a paracrine manner via stimulating the release of IL-1, TNFα, or IFNγ from OFs or via activation of these cytokine receptors. Smoking is involved other aspects of the innate immune system, such as via nuclear factor-κB signaling (38) and is associated with increased soluble ICAM concentrations, and soluble ICAM levels are higher in TAO patients than in Graves' patients without TAO or in controls (39). Smoking also appears to play a role in the adaptive immune system in autoimmune thyroid disease, being a risk factor for Graves' disease, although the odds ratio is approximately half that associated with TAO (40, 41), and possibly being protective for Hashimoto's thyroiditis (42). One of the emerging mechanisms by which cigarette smoke appears to be acting in other smoking-related conditions such as chronic obstructive pulmonary disease is by stimulation of macrophages. In macrophages from patients with chronic obstructive pulmonary disease, CSE can stimulate the release of proinflammatory cytokines including TNFα (15). In certain situations, such as mucin production from airway epithelial cells, the action of cigarette smoke is also synergistic with cytokine action (43). In contrast, in this fibroblast TAO model, cigarette smoke appears to be having direct and specific actions on fibroblast function that is independent of, but can be synergistic with, certain cytokine-mediated effects. If cigarette smoke can also stimulate macrophages and other cells involved in the acute inflammation in TAO to secrete proinflammatory cytokines into the orbit, this could be an additional and complementary mechanism by which smoking might worsen TAO.

The effects of smoking in TAO are likely to be complex, with influences on both the innate and adaptive immune system as well as direct actions upon fibroblast function. The identity of the active compound(s) in cigarette smoke, the molecular mechanisms of the actions of cigarette smoke in TAO, and the synergy with IL-1 remain to be studied. The identification and dissection of the numerous actions of the compounds in cigarette smoke, including determining how the respective signal-

ing pathways interact may give additional insight into how the environment (smoking) affects autoimmune disease.

These *in vitro* observations of the effects of cigarette smoke on a model of TAO provide a plausible and novel explanation of the biological link between cigarette smoking and TAO and have potential therapeutic implications. The synergy between cigarette smoke and cytokine action may have relevance to other autoimmune and inflammatory conditions where the link with cigarette smoking is also well known but poorly understood.

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