Table 3. Association between urine oxytocin and social capital indicators by gender.

		Female (n = 50)					Male (n = 31)					P for interaction between gender and social capital variables*
			Crude		Adjusted*			Crude		Adjusted*		
		Mean OT level (SD) unit: μU/ml per creatinin g/L	B	95% CI	B	95% CI	Mean OT level (SD) unit: μU/ml per creatinin g/L	B	95% CI	B	95% CI	
Cognitive social capital												
Social trust	High	83.4 (19.7)	ref		ref		116.2 (57.7)	ref		ref		0.29
	Middle	112.9 (41.3)	**29.5**	**(2.5 to 56.5)**	**29.0**	**(1.4 to 56.5)**	112.4 (31.6)	−3.8	(−44.6 to 37.0)	−2.1	(−46.8 to 42.7)	
	Low	121.3 (34.2)	**37.8**	**(5.7 to 70.0)**	**33.5**	**(0.3 to 66.7)**	110.9 (48.9)	−5.3	(−5.3 to 39.6)	−7.9	(−62.2 to 46.4)	
	P for trend		**0.024**		**0.046**			0.81		0.77		
Mutual aid	High	95.9 (31.0)	ref		ref		116.2 (57.7)	ref		ref		0.38
	Middle	102.7 (34.5)	6.8	(−19.2 to 32.8)	4.8	(−22.0 to 31.6)	110.7 (31.2)	−5.5	(−45.8 to 34.9)	−3.1	(−47.8 to 41.6)	
	Low	127.5 (42.9)	**31.6**	**(4.1 to 59.1)**	28.7	(−0.6 to 57.9)	114.1 (51.2)	−2.1	(−48.2 to 44.0)	−5.5	(−58.8 to 47.8)	
	P for trend		**0.021**		**0.046**			0.94		0.83		
Structural social capital												
Community participation	2 or more	103.7 (33.8)	2.3	(−23.3 to 27.9)	12.0	(−16.1 to 40.1)	106.8 (32.9)	−18.4	(−56.4 to 19.7)	−17.7	(−57.3 to 21.9)	0.34
	1	124.5 (45.5)	23.1	(−5.0 to 51.3)	**29.8**	**(1.1 to 58.6)**	105.2 (44.7)	−20.0	(−58.0 to 18.0)	−39.0	(−89.7 to 11.6)	
	0	101.4 (34.9)	ref		ref		125.2 (47.7)	ref		ref		
	P for trend		0.99		0.52			0.32		0.35		

*Adjusted for age, number of children, self-rated health, and education.

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sured using the alkaline picrate colorimetric method (modified Jaffe).

Assessment of Social Capital

Individual perceptions of community social capital were assessed within both cognitive and structural domains, following the approach most widely practiced in the previous literature [29,30,38,39]. Indicators of cognitive social capital included items measuring social trust and mutual aid. Social trust was assessed with a single item: "Do you think that people in your neighborhood trust each other?" with 4-Likert-scale responses of "yes", "somewhat yes", "somewhat no", and "no". This measurement of social trust is somewhat different from standard measures, such as "In general, would you say that your neighbors can be trusted?" [40]. However, by asking only about whether the individual believe neighbors can be trusted, the latter question cannot measure perceived community norms in terms of mutual trust because it captures only an individual's perception of the trustworthiness of his or her neighbors and not how trustworthy neighbors might perceive each other to be [34,41]. Thus, we used a different question to measure the perceived norms of social trust in the community, which has also been used in previous studies [29,30,38,39]. The responses "relatively no" and "no" were collapsed a priori in order to create three categories, as the "no" group was too small (4.3%) by itself to permit further statistical analysis; thus, the response distribution across categories was high trust (20.4%), middle trust (53.8%), and low trust (25.8%). Mutual aid was assessed with a single item: "Do you think that people in your neighborhood aid each other?" with 4-poin Likert-scale responses of "yes", "somewhat yes", "somewhat no", and "no" as used in previous studies [29,38,39]. The responses "somewhat no" and "no" were collapsed a priori in order to create three categories, as the "no" group was too small (6.5%) by itself to permit further statistical analysis; thus, the response distribution across categories was high mutual aid (23.7%), middle mutual aid (45.2%), and low mutual aid (31.2%). The distribution of responses were quite similar with those obtained in a previous study using a community representative sample of middle-aged women in Japan [39].

Structural social capital was assessed by asking the participants about their community participation, which was calculated as the number of organizations in which the respondent reported participating; this measure has been used in previous studies to show the association with health [39,42]. Organizations with which participants reported being involved included child rearing circles, parent-teacher associations, civic organizations, consumers' cooperative societies, unions/religious groups, or other community groups. On the basis of the distribution of responses, we categorized community participation into three groups: no participation (38.3%), 1 organization (27.7%), and 2 or more organizations (34.0%). Community participation was lower in our sample than in a previous study using a community representative sample of middle-aged individuals in Japan (no participation [23.5%], 1 organization [23.2%], and 2 or more organizations [53.4%]) [39], probably because our sample had younger children.

Covariates

Covariates include age, number of children, self-rated health (5-point Likert scale), and education (high school or less, some college, and college or more). All were measured via questionnaire.

Statistical Analysis

Associations between cognitive and structural social capital and urinary OT level were analyzed using regression models stratified by gender. First, bivariate models were performed to determine crude association. Then, multivariate models were performed with adjustment for age, number of children, self-rated health (5-point Likert scale), and education (high school or less, some college, and college or more) to see the independent association between social capital and OT. Further, we tested for an interaction effect between gender and social capital on OT. All analyses were performed using the STATA MP version 12.0 software package (STATA Corporation, College Station, TX, 2011).

Results

Table 1 shows the distribution of demographic and social capital indicators among participants by gender. Basically, participants were middle-aged (mean for women: 35.9 [SD: 3.9] years, men: 36.9 [SD: 2.8] years), more than 90% of them reported their health as being good or better, and the majority graduated high school. Levels of social trust were higher among women than men: 22% of women reported low social trust, while 29% of men reported the same. Higher community participation was observed among women than men (i.e., 42% and 32% of them participated 2 or more organization, respectively).

OT levels for both men and women are described in Table 2. The mean (SD) OT levels were similar between women and men: 108.9 (38.2) and 112.8 (42.1) μU/mL per creatinine g/L, respectively; t-test indicated these results were not significantly different ($p>0.6$).

Table 3 and Figure 1 show the association between OT and social capital indicators. Among women, an inverse association between social trust and OT level was observed: mean OT level of women who perceived high, middle, and low social trust were 83.4, 112.9, and 121.3 μU/mL per creatinine g/L, respectively, showing a significant inverse dose-response association (p for trend $= 0.024$). This association remained significant even after adjustment for covariates (age, number of children, self-rated health, and education, $p = 0.046$). Similarly, women who perceived low mutual aid showed higher OT level than those who perceived mutual aid as middle or high ($p = 0.021$); these effects remained significant in the adjusted model ($p = 0.046$). However, the association between OT and structural social capital (i.e., community participation) was not linear: the mean OT levels of women who participated in 2 or more, 1, or 0 organizations were 103.7, 124.5, or 101.4 μU/mL per creatinine g/L, respectively, suggesting an inverse-U shape association. In the adjusted model, women who participated in 1 organization showed OT levels 29.8 μU/mL per creatinine g/L higher than women who did not participate in any organization, a significant effect ($p = 0.042$). By contrast, no association was found between OT and social capital indicators among men ($p>0.3$).

Further, the interaction effect of gender and social capital on OT was considered. However, we found no interaction effect between gender and social capital on OT ($p>0.2$, Table 2), probably due to small sample size.

Discussion

The current study revealed that among women, OT levels measured in the urine –a proxy of circulating peripheral OT level– was inversely associated with cognitive social capital: those perceiving lower social trust showed higher OT levels than those perceiving high social trust. Interestingly, OT level showed an inverse U-shaped association with structural social capital, with women participating in 1 organization showing higher OT levels than women participating in either 0 or 2 or more organizations. Among men, no association was observed between OT and

indicators of social capital. However, because of the study's cross-sectional design, our findings cannot provide insight into the causal direction of effects; only an association between OT and social capital can be confirmed.

To the best of our knowledge, this is the first study that has considered the association between OT levels and social capital. Previous studies have reported that baseline plasma OT was positively associated with relationship stress among young women [43] but inversely associated with frequency of social contacts among postmenopausal women [44]. Thus, it is possible that exposure to a community with low social trust or mutual aid induces relationship stress or less frequent contact with neighbors, resulting in elevations in circulating OT levels. It has also been reported that smaller social networks lead to higher levels of stress biomarkers (e.g., cortisol [45]) and inflammation marker (e.g., IL6-receptor and C-reactive protein [46]), although social networks and social capital are not exactly the same.

Our study showed an inverse association between OT and social trust, although other studies reported positive association between plasma OT and general trust [6,10]. One possible explanation for this discrepancy is that urinary and plasma OT levels might show different effects of trust. For example, Feldman et al. reported that urinary OT was associated with interactive stress, while plasma OT was associated with affect synchrony [31]. Another possible reason for this difference might be that we investigated the general concept of trust between neighbors without directly asking whether the respondents trust any particular neighbor. In other words, we measured the respondents' perception of community, which they could interpret as a stressor, not as a proxy of their tendencies towards trust in others (i.e. general trust). Women who perceived low social trust might have higher levels of community-related stressors, and chronic exposure to such stress from community sources might elevate circulating OT levels, as indicated by other studies [43,44]. Alternatively, women who perceived low social capital within their community might be more likely spend time with their families to avoid contacts with neighbors, which in turn could lead to more positive experiences with parenting or romantic relationships with partner, both of which enhance OT [8,9].

The reasons for the inverse U-shaped association observed between structural social capital and urinary OT among women are unknown. However, it might be that participating in one organization is a stressor for women–especially during child raising–because the women might be unwilling or uninterested participants in the organization, only participating because of the duty to make friends for their children or receive useful information for child rearing. Women who participate in more organizations may find it to be a positive experience, whereas those who participate in only one may tend to do so out of obligation, therefore finding it less satisfying or more stressful. Further study is needed to replicate and understand the inverse U-shaped association between structural social capital and OT among child-rearing populations in other settings.

Among men, OT was not significantly associated with indicators of social capital, perhaps because of the smaller number of enrolled men than women and resulting lower power to detect effects. However, this result is consistent with those of previous studies, which have found weaker effects of OT on blood pressure and norepinephrine [32]–which are also linked with stress–in men. It is possible that even if men perceived low social capital in the community or participated in community organizations, this is not a stressor for them in the same way as it is for women; this is plausible given other research suggesting that community social capital is less important–and work-place social capital more important–for men than women. A previous study reported that workplace social capital was associated with hypertension among male, but not women [47]. Therefore, it is somewhat less surprising that OT levels did not differ according to perceived social capital among men. Further research is needed to elucidate the association between OT and workplace social capital among men.

Several limitations need to be addressed before drawing firm conclusions. First, the participants were a convenience sample and limited to middle-aged adults with children; this precludes the generalizability of the findings. However, our sample was selected from various locations in greater Tokyo and covers a wide range of social capital and socioeconomic status. Second, we used spot urine samples as a proxy of circulating OT, but urinary OT might be different from plasma OT, saliva OT (i.e. peripheral OT) or cerebrospinal fluid OT (i.e. central OT) [31,48]. Further study is needed to replicate these findings using other OT measurement in order to determine whether other forms of OT may yield different insights into the underlying biological processes related to trust. Third, our cross-sectional design precludes conclusions about the causal relationship between social capital and OT, that is, whether higher OT causes low social capital or vice versa. Future prospective study is needed to confirm whether living in low-social-capital community elevates circulating OT levels.

In conclusion, we found an inverse association between urinary OT and cognitive social capital among women. Structural social capital showed an inverse U-shaped association with OT among women. Having low social capital might be a stressor for women who are raising children, as shown by their high OT levels. No association was found between OT and social capital among men. Further study using prospective design and other peripheral or central OT measurements is warranted to confirm whether having low social capital elevates levels of circulating OT.

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Author Contributions

Conceived and designed the experiments: TF. Performed the experiments: TF. Analyzed the data: TF. Contributed reagents/materials/analysis tools: TF LDK KM IK. Wrote the paper: TF LDK KM IK.

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Factors Associated with Steroid Phobia in Caregivers of Children with Atopic Dermatitis

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Abstract: **Topical corticosteroids (TCS) are first-line therapeutic agents for atopic dermatitis (AD). Some patients express irrational fear and anxiety about using TCS, which leads to poor outcomes for AD. Although it is important to understand the factors underlying steroid phobia so that its effects can be minimized, few studies have addressed this subject. Here, we used a questionnaire to investigate predictive factors for steroid phobia in the caregivers (usually mothers) of children with AD. We studied 436 children with AD (mean age 47.6 mos, range 2–236 mos) who newly visited our AD outpatient unit. The caregivers were asked to complete a medical history questionnaire regarding AD. Steroid phobia was analyzed for correlations with other patient and caregiver variables. Overall, 38.3% of the caregivers were reluctant to use TCS on their children's skin. Patient characteristics female sex (odds ratio [OR] = 1.85 vs male; p = 0.005), child's paternal history of AD (OR = 1.94; p = 0.03), and frequent changing of clinics (OR = 1.25; p = 0.03) were predictive factors for steroid phobia. AD severity did not correlate with steroid phobia. Our findings suggest that greater attention to the patient's sex and clinical background of patients with AD is important to the success of AD therapy, regardless of AD severity.**

Topical corticosteroids (TCS) are first-line therapeutic agents for atopic dermatitis (AD) (1). In daily clinical practice, it is common for patients to express irrational fear and anxiety about using TCS (steroid phobia) (2–6). Steroid phobia may lead to poor patient adherence to TCS therapy, resulting in poor control of AD (3,5), and poor control of AD may lead to physical, psychological, and social isolation, including sleep disturbance, teasing, and school refusal, which are thought to be more serious problems than the adverse effects of TCS (3,7).

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Furthermore, those problems result in lower quality of life, not only for children with AD, but also for their families (8), sometimes even leading to family disruption (3). Some patients and caregivers with steroid phobia choose alternative and unproven therapies, which may cause exacerbation of AD (9). Others choose strict dietary therapy that results in malnutrition or failure to thrive (10).

In Japan, steroid phobia is widespread because of confusion and misinformation regarding AD therapies, including strict dietary restriction therapy, in the 1980s and negative media publicity that has exaggerated the adverse effects of TCS since the 1990s (11). Moreover, alternative and unproven therapies have gained popularity as an "atopy industry" (12). Incorrect information has often been disseminated over the Internet (13). In addition to these problems, Japan has experienced scams and lawsuits regarding alternative therapies, deaths due to malnutrition caused by extreme dietary restriction therapy, teasing-linked suicides, and at worst, family suicides (13).

Although steroid phobia is a serious problem, few studies have investigated the factors associated with it (14). The present study was designed to shed light on this, with the ultimate objective of improving the treatment of AD.

MATERIALS AND METHODS

Patients

The present study was conducted on new outpatients (and their caregivers) with AD aged 1 month to 20 years of age who visited the Outpatient Unit of the Division of Allergy, National Center for Child Health and Development, Tokyo, from April 2003 to June 2006. Patients were excluded if they had other forms of inflammatory dermatitis. The hospital ethics committee of the National Center for Child Health and Development approved this study, and it was conducted in accordance with the principles outlined in the Declaration of Helsinki.

Clinical Background and Data

Allergists diagnosed AD. The severity of AD in each patient was assessed using the scoring of atopic dermatitis (SCORAD) index (15) and then classified into one of four categories according to that index (remission 0, mild 1–25, moderate 25–49, and severe ≥50) (16). Age, sex, age at onset of AD, duration of eczema, parental history of AD, current usage of TCS, and results of blood tests (eosinophil and total immunoglobulin E [IgE] levels) were confirmed from the medical records for each patient.

Questionnaire

The caregiver attending a child patient—in most cases (approximately 95%) the mother—was asked to complete a questionnaire before the doctor examined the child. The questionnaire consisted of items concerning perceptions regarding TCS and the history of AD therapy. The items regarding perceptions of TCS included caregiver's steroid phobia, adverse effects, and perceived image of TCS therapy. Steroid phobia was assessed by asking "Would you agree to use TCS on your child's skin?" Those who answered "never" or "no, I'd rather not use TCS if I can avoid it" were defined as having steroid phobia. Preconceptions regarding adverse effects and the image of TCS therapy were ascertained by asking open-ended questions. The history of AD therapy was also ascertained using open-ended questions, and frequency of changing clinics, use of alternative therapies, and negative experiences with doctors were determined. Caregivers were also asked about the history of nonadherence to TCS therapy, how they applied TCS to the child, and the use of soap to bathe the child (Table 2).

Statistical Analyses

To check the validity of the single-item question about steroid phobia, the chi-square test was used to analyze for associations between the question "Would you agree to use TCS on your child's skin?" and the caregiver's image of TCS, history of nonadherence to TCS therapy, the caregiver's preconceptions regarding adverse effects, application of TCS, and use of alternative care. Then we performed bivariate logistic analysis of steroid phobia against patient sex, age, onset age, duration of eczema, severity of AD, parental history of AD, eosinophil count, total IgE level, frequency of changing clinics, and negative experience with doctors. Finally, we performed multiple logistic analysis to identify factors associated with steroid phobia, using as explanatory variables the factors that showed a marginal association with steroid phobia with $p < 0.4$ in the bivariate analysis. Data were analyzed using STATA software (Windows version 8.0; StataCorp, College Station, TX). $p < 0.05$ was considered to indicate statistical significance in all comparisons. The postestimated goodness-of-fit (Hosmer-Lemeshow) was confirmed for logistic regression analysis.

RESULTS

Patients

Four hundred forty-eight caregivers completed the questionnaire. We excluded 12 patients who had other

forms of inflammatory dermatitis, leaving 436 patients for the analyses: 286 male (65.6%) and 150 female (34.4%). Their mean age was 47.6 ± 48.9 months (range 2–236 mos). The characteristics of the patients are summarized in Table 1.

Confirmation of the Single-Item Question on Steroid Phobia

The incidence of steroid phobia measured using a single-item question was 38.3% of all caregivers (Table 1) and 58.7% of those with a history of nonadherence to TCS therapy (Table 2). We used the chi-square test to confirm the validity of the single-item question about steroid phobia, "Would you agree to use TCS on your child's skin?" There were strong correlations between negative perceptions regarding TCS (negative image of TCS, history of nonadherence to TCS therapy, strong apprehension regarding adverse effects of TCS, not a current user of TCS, and preference for alternative care) and the question (Table 2). Because these negative perceptions of TCS are a crucial component of steroid phobia, we considered this single-item question about steroid phobia to be useful.

Bivariate Analyses

In the bivariate analyses, female sex (odds ratio [OR] = 1.59, 95% confidence interval [CI] = 1.06–2.39), duration of eczema (OR = 0.93, 95% CI = 0.88–0.99), and paternal history of AD (OR = 1.91, 95% CI = 1.06–3.44) were significantly associated with steroid phobia; severity of AD and blood sample data were not (Table 3).

Multivariate Analyses

To evaluate the effects of confounding factors, a logistic regression model was adjusted for patient sex, age, duration of eczema, parental history of AD, frequency of changing clinics, and negative experience with doctors. In the multivariate analyses, female sex (adjusted OR [aOR] = 1.85, 95% CI = 1.20–2.85), child's paternal history of AD (aOR = 1.94, 95% CI = 1.03–3.58), and frequent changing of clinics (aOR = 1.25, 95% CI = 1.03–1.53) were significantly associated with steroid phobia (Table 4).

DISCUSSION

We found that the predictive factors for steroid phobia in caregivers of children with AD are patient female sex,

TABLE 1. *Characteristics of Patients*

	Mean (range) or	SD or %
Demographic		
Patient's sex M/F	286/150	65.6/34.4
Patient's age (months)	47.6 (2–236)	48.9
< 12	120	27.5
12–72	217	49.8
> 72	99	22.7
Clinical characteristic		
Onset age	10.4 (1–191)	21.3
< 12	338	77.5
12 < < 36	74	17.0
36 <	16	3.7
Duration of eczema	36.8 (0–224)	43.3
Severity of (SCORAD) index		
Remission (0)	12	2.8
Mild (1–25)	159	36.5
Moderate (> 25)	175	40.1
Severe (> 50)	90	20.6
Parental history of	102	23.4
Mother	60	13.8
Father	51	11.7
Both	9	2.1
Eosinophil count (/μL)	662.3 (0–5,592.4)	689.5
< 294	93	
294–467	93	
467–793	93	
> 793	93	
Eosinophil count (%)	6.74 (0–34.8)	4.86
< 3.55	93	
3.55 < < 5.7	95	
5.7 < < 8.7	92	
8.7 <	92	
Total IgE (IU/mL)	2259.2 (2–97,600)	8692
< 53.9	93	
53.9 < < 310	93	
310 < < 1,141	93	
1,141 <	94	
Steroid phobia		
Caregivers who were reluctant to use	167	38.3
Past consultation for AD		
Frequency of changing	2.1 (0–6)	1.2
Caregivers who had negative experiences with	80	18.4
Application of TCS		
Use of TCS		
Non-current	24	5.5
Past user	64	14.7
Current user	334	76.6
Caregivers who apply TCS sparingly at doctor's	171	39.2
Caregivers who apply TCS sparingly at own	81	18.8
Caregivers who apply TCS liberally at doctor's	58	13.3
Caregivers who apply TCS liberally at own	35	8
Alternative		
Caregivers who preferred alternative	71	16.3
Caregivers who don't use soap to bathe the child	25	5.7

AD, atopic dermatitis; SCORAD, Scoring Atopic Dermatitis; TCS, topical corticosteroids.

TABLE 2. *Confirmation of Single-item Question About Steroid Phobia*

Category	Steroid phobia (+) (N = 167)	Steroid phobia (−) (N = 262)	p-Value†
Image of TCS held by caregiver			
Negative	96 (62.3)	88 (37.1)	<0.001*
Positive	7 (4.5)	65 (27.4)	
Both negative and positive	50 (32.5)	75 (31.6)	
Other	1 (0.6)	9 (3.8)	
History of non-adherence with TCS (by caregiver)			
Yes	90 (57.7)	63 (25.0)	<0.001*
No	66 (42.3)	189 (75.0)	
Preconceptions regarding adverse effects of TCS (by caregiver)			
Adverse effects on the skin (including skin thinning and darkening)			
Yes	112 (67.1)	128 (48.9)	<0.001*
No	55 (32.9)	134 (51.1)	
Skin thinning			
Yes	54 (32.3)	59 (22.5)	0.024*
No	113 (67.7)	203 (77.5)	
Skin darkening			
Yes	35 (21.0)	28 (10.7)	0.003*
No	132 (79.0)	234 (89.3)	
Systemic adverse effects of TCS			
Yes	57 (34.1)	53 (20.2)	0.001*
No	110 (65.9)	209 (79.8)	
Application of TCS			
Use of TCS			
Non-current user	11 (6.7)	10 (4.0)	<0.001*
Past user	41 (25.2)	23 (9.1)	
Current user	111 (68.1)	219 (86.9)	
Caregivers who apply TCS sparingly at doctor's instruction	66 (50.0)	105 (49.3)	0.16
Caregivers who apply TCS sparingly at own judgment	38 (28.8)	43 (20.2)	
Caregivers who apply TCS liberally at doctor's instruction	17 (12.9)	41 (19.2)	
Caregivers who apply TCS liberally at own judgment	11 (8.3)	24 (11.3)	
Alternative care			
Caregivers who preferred alternative care			
Yes	37 (22.2)	34 (13.0)	0.013*
No	130 (77.8)	228 (87.0)	
Caregivers who don't use soap to bathe the child			
Yes	8 (4.9)	17 (6.6)	0.46
No	156 (95.1)	240 (93.4)	

*p < 0.05.
†Chi-square test.
Data given in parentheses are expressed as percentage.

child's paternal history of AD, and frequent changing of clinics for the patient but not severity of AD. These results suggest that greater attention to the clinical background of patients with AD is important in addressing steroid phobia, regardless of the severity of AD.

The incidence of steroid phobia among caregivers was 38.3% (Table 1), which is consistent with previous studies. A report from Hong Kong showed that 40% of patients with moderate and 60% of patients with severe AD expressed concern about using TCS, but there was no association between steroid phobia and severity of AD (6). In the United Kingdom, 72.5% of patients with AD worried about using TCS, and 36.5% of those had been nonadherent to TCS therapy (4). Of caregivers of children with AD in Australia, 40% answered that TCS was dangerous, and 20% said it was too dangerous to use on their child's skin (17). In France, 80.7% of the parents of children with AD and people with AD reported having fears about TCS, and 36% admitted nonadherence to treatment (18). Although methodologic differences make it difficult to compare these percentages directly, approximately one-third of parents are reluctant to use TCS on their children. Medical care providers need to be sensitive to this anxiety about using TCS in their daily clinical practice.

We also confirmed the validity of the single question about steroid phobia, "Would you agree to use TCS on your child's skin?" by finding strong correlations with negative perceptions regarding TCS (negative image of TCS, history of nonadherence to TCS therapy, strong apprehension regarding adverse effects of TCS, not a current user of TCS and preference for alternative care) (Table 2). This result was similar to a recent study that reported a correlation between steroid phobia and the need for reassurance, the belief that topical corticosteroids

TABLE 3. *Correlation with Steroid Phobia (Bivariate)*

	Bivariate OR	95% CI	p-Value
Patient's sex			
Male	Reference		0.025*
Female	1.59	1.06–2.39	
Patient's age (mos)			
< 12	Reference		0.027*
12–72	1.06	0.67–1.67	
> 72	0.54	0.30–0.96	
Onset age (mos)			
< 12	Reference		0.87
12–36	1.14	0.69–1.90	
> 36	1.05	0.38–2.88	
Duration of eczema (yrs)	0.93	0.88–0.99	0.018
Severity of AD (SCORAD index)			
Remission (0)	Reference		0.8
Mild (1–25)	0.59	0.18–1.90	
Moderate (25–50)	0.63	0.20–2.04	
Severe (> 50)	0.7	0.21–2.33	
Parental history of AD			
Mother			
No	Reference		0.39
Yes	1.28	0.73–2.22	
Father			
No	Reference		0.031*
Yes	1.91	1.06–3.44	
Eosinophil count (/μL)			
< 294	Reference		0.82
294–467	0.89	0.49–1.63	
467–793	0.86	0.47–1.58	
793 <	1.12	0.62–2.03	
Eosinophil count (%)			
< 3.55	Reference		0.51
3.55–5.7	0.75	0.40–1.38	
5.7–8.7	1.06	0.58–1.92	
> 8.7	1.16	0.64–2.10	
Total IgE (IU/ml)			
< 53.9	Reference		0.81
53.9–310	0.75	0.41–1.37	
310–1,141	0.93	0.51–1.68	
> 1,141	0.91	0.50–1.65	
Past consultation for AD			
Frequency of changing clinics	1.14	0.96–1.34	0.13
Caregivers with negative experience with doctors			
No	Reference		0.14
Yes	1.45	0.89–2.37	

*p < 0.05.

TABLE 4. *Correlation with Steroid Phobia (Multivariate)*

	†Multivariate OR	95% CI	p-Value
Patient's sex			
Male	Reference		0.005*
Female	1.85	1.20–2.85	
Patient's age (mos)			
< 12	Reference		
12–72	1.03	0.62–0.73	0.9
> 72	0.55	0.20–1.53	0.25
Duration of eczema (years)	0.96	0.86–1.07	0.45
Parental history of AD			
Mother			
No	Reference		0.39
Yes	1.15	0.64–2.05	
Father			
No	Reference		0.034*
Yes	1.94	1.03–3.58	
Past consultation for AD			
Frequency of changing clinics	1.25	1.03–1.53	0.026*
Caregivers with negative experience with doctors			
No	Reference		0.78
Yes	1.08	0.63–1.85	

*p < 0.05.
†Adjusted for all values listed above.

pass through the skin into the bloodstream, a prior adverse event, inconsistent information about the quantity of cream to apply, a desire to self-treat for the shortest possible time, or poor treatment adherence (18). Our observation suggests that appropriate education of patients to remedy negative perceptions of TCS would be one good strategy for addressing steroid phobia, as others have noted (2,4,19).

In this study, most patients and caregivers (91.3%) had used TCS, at least in the past (Table 1), which suggests that only a few caregivers had totally rejected TCS therapy for their child at the onset of AD. This implies that, whether the caregivers of children with AD become steroid phobic or not depends on the experience during AD therapy that they have received. An earlier study on factors related to steroid phobia suggested that a preconception of ineffectiveness or adverse effects of TCS was associated with steroid phobia (14).

Our results indicate that the patient's sex, a paternal history of AD, and frequent changing of clinics for the patient were associated with steroid phobia (Table 4).

There have been no studies focusing on patient sex as an association factor for steroid phobia. In our study, we found that misinterpretation of skin darkening as a TCS side effect was significantly associated with steroid phobia (Table 3). Light skin, bright eyes, and black hair have long been considered essential factors for beauty in Japanese girls (20). Thus, we assume that our finding may be due to a cultural factor in Japan that—together with the misinterpretation—leads the parents of girls to be reluctant to use TCS for their children.

We found that the child's paternal—but not maternal—history of AD was associated with steroid phobia (Tables 3 and 4). If parents had experienced treatment failure in the past, they tended to feel steroid phobia for their children. On the other hand, parental history of AD could be a remedy for steroid phobia because they may have been advised about or read correct knowledge regarding TCS during their previous experience with TCS therapy. We should have asked whether the parents had experienced treatment failure in the past, but in Japan, steroid phobia was widespread in 1980s (12). Therefore some parents with AD had not received

appropriate or successful TCS therapy and might have negative perceptions of TCS. Although our results showed that one of the predictive factors for steroid phobia was child's paternal history of AD, this might be because the caregivers of most Japanese children with AD are their mothers. Mothers would have more chances to acquire the correct knowledge regarding TCS from doctors, and any steroid phobia they might have might be relieved. On the other hand, fathers would have less opportunity to be in contact with their children's doctor, and their steroid phobia might remain. Family or relatives have also been reported to be major sources of steroid phobia (4,6,9). Thus, a father with a history of AD might cause steroid phobia in the mother–caregiver. We believe that these results indicate that correct information regarding TCS is needed not only for mother–caregivers, but also for father–partners.

Frequent changing of clinics suggests a history of treatment failure or distrust of medical care services, because in Japan patients can go to clinics without any referral. This finding of frequent changing of clinics might indicate that negative experiences with doctors, including ineffectiveness of TCS, are associated with steroid phobia.

On the other hand, AD severity was not associated with steroid phobia, which is consistent with previous studies (6,18). Furthermore, as others have noted (14,21), in our study, personal negative experiences and attitudes such as a preconception of ineffectiveness might have been more important as factors underlying steroid phobia than objective factors such as AD severity evaluated by a doctor. This finding suggests that AD severity should not be a factor in evaluating whether a patient has steroid phobia.

This study has a number of limitations. First, it was conducted at a national medical center where most patients would know that doctors would use TCS for AD therapy. Therefore, strongly phobic patients might have been underrepresented. Further studies of such patients should be undertaken to compare with our results. Second, the questionnaire about steroid phobia was not validated in other studies (4,6,17,18), although we confirmed that a single-item question, "Would you agree to use TCS on your child's skin?" was associated with negative perceptions of TCS (Table 2). Although we tried to identify negative experiences with doctors using open-ended questions regarding history of AD therapy, a future study should have multiple-choice questions and focus on a history of treatment failure and the patient–doctor relationship. Third, because of the cross-sectional study design, the presumed cause-and-effect relationship between predictive factors and steroid phobia may be the reverse; for example, steroid phobia may have been the reason for frequent changing of clinics. Fourth, other unmeasured factors, such as parental level of education, social status, and personality, might have confounded the results. Further studies should be done regarding these factors in steroid phobia, although medical expense would not be burden for Japanese caregivers because there are medical care subsidies for children in Japan.

In conclusion, our study suggests that greater attention to patient sex, paternal history of AD, and frequency of changing clinics for the patient will aid physicians in addressing steroid phobia, whereas AD severity does not play any role. It is necessary to devote sufficient time to careful elucidation of the clinical background of patients with AD from their caregivers. This would help physicians understand any steroid phobia of caregivers and contribute to overcoming steroid phobia in patients with AD and their caregivers.

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Salivary Cortisol Response to Stress in Young Children with Atopic Dermatitis

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Abstract: **Poor responsiveness of the hypothalamic–pituitary–adrenal (HPA) axis under stress may be one explanation for stress-induced exacerbation of atopic dermatitis (AD) symptoms. In previous studies, children and adults with AD showed attenuated salivary cortisol responses to psychosocial stress, suggesting hyporesponsiveness of the HPA axis, but few studies have been conducted in young children, who are vulnerable to systemic side effects of topical corticosteroid (TCS) therapy. We evaluated whether salivary cortisol responses to the stress of venipuncture in young children with AD were related to the severity of AD or performance of TCS therapy. We studied 38 young children with AD (median age 16.5 mos, range 3–66 mos) being treated at our outpatient unit. Patients were divided into three groups according to the scoring of atopic dermatitis index: mild (n = 12), moderate (n = 14), and severe (n = 12). To evaluate the responsiveness of the HPA axis to stress, salivary cortisol was determined before and after venipuncture. Salivary cortisol responsiveness to stress correlated negatively with severity of AD (p = 0.048) but not with previous use of TCS (p = 0.43) in young children with AD. Our findings suggest that the disease activity of AD, rather than TCS use, is responsible for HPA axis dysfunction in children with AD.**

Clinical observations and experimental findings have emphasized that exacerbation of atopic dermatitis (AD) symptoms is closely related to psychosocial stress (1,2). Stress itself can reportedly cause epidermal barrier dysfunction and mast cell activation through release of neuropeptides, which in turn facilitates exacerbation of allergic inflammation (reviewed in reference 1). Stress also activates the hypothalamic–pituitary–adrenal (HPA) axis to release cortisol, a potent attenuator of inflammatory reactions in general, although previous studies indicated that children and adults with AD showed attenuated salivary cortisol responses to psychosocial stress, suggesting hyporesponsiveness of the HPA axis (3,4). Therefore, poor responsiveness of

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the HPA axis under stress may be one explanation of stress-induced exacerbation of AD symptoms. Similar endocrine irregularities of the HPA axis have also been reported after pharmacologic challenge in patients with AD (5,6). It may be argued that hyporesponsiveness of the HPA axis in patients with AD is simply a side effect of topical corticosteroid (TCS) therapy, which may lead to steroid phobia (fear of TCS use) in patients with AD and even in health care professionals (7). Previous reports have investigated the influence of TCS on the HPA axis in children with AD, with contradictory results (8–10). Other studies have suggested that AD disease activity, rather than the use of TCS, is responsible for changes in the HPA axis in patients with severe AD (5,11). Young children are believed to be at special risk for systemic effects of TCS because of high percutaneous absorption of TCS due to their relatively large body surface area per weight. Therefore, it is important to investigate whether HPA dysfunction is observed in young children with AD and is related to TCS therapy.

Blood cortisol concentration increases in response to unpredictable, uncontrollable, and novel situations (12,13). Salivary cortisol levels correlate with plasma unbound cortisol levels (14). Moreover, because saliva samples can be obtained without stress, salivary cortisol assessment is a reliable tool for investigating HPA axis function, especially in young children (13). Previous studies reported a significantly attenuated cortisol response to standardized laboratory psychological stress, which consisted mainly of public speaking and mental arithmetic tasks in front of an audience, in children and adults with AD (3,4), but those procedures are difficult to implement in children younger than 6 years of age. Moreover, venipuncture is an unpredictable, uncontrollable, novel situation for young children, so it itself might be a significant stressor resulting in activation of the HPA axis in young children, detected as high cortisol levels. We used venipuncture as the acute stressor to investigate changes in the level of salivary cortisol.

We evaluated whether impairment of the salivary cortisol response to the stress of venipuncture in young children with AD was related to the severity of the AD symptoms and whether TCS use affected the response.

METHODS

Subjects

We studied 38 young children (24 boys and 14 girls) with AD (median age 16.5 months, range 3–66 months) being treated at our outpatient unit. Patients who had received regular TCS therapy were included, but those who had received inhaled or oral corticosteroids in the preceding 6 months were excluded. All patients were Japanese whose parents were of middle socioeconomic status. An allergist made the diagnosis of AD in accordance with the clinical criteria defined by the Japanese Dermatological Association (15). The severity of AD in each patient was assessed using the scoring of atopic dermatitis (SCORAD) index (16). The severity of AD was defined according to the SCORAD index (mild <25, moderate 25–50, severe ≥ 50).

Previous use of TCS was assessed by a modification of the previously defined score, the treatment score (Table 1) (10), which was based on the potency of the preparation used (TCS preparations were grouped according to clinical potency, as described in the Japanese Therapeutic Guideline for AD (17)), percentage of body surface area to which it was applied, the duration of treatment in the last month. The hospital ethics committee of the National Center for Child Health and Development approved this study. Informed consent was obtained from all caregivers.

Procedures

To evaluate the responsiveness of the HPA axis to stress, salivary cortisol concentrations were determined before and after venipuncture. Venipuncture for clinical purposes and saliva sampling were performed between 10:00 A.M. and 3:00 P.M. to avoid any possible inhibitory effect of a high morning basal cortisol level on further cortisol release (18). To determine free cortisol concentration, saliva samples were collected 5 minutes before and 15 to 20 minutes after the venipuncture was completed, because that is when salivary cortisol level peaks (13).

Cortisol Measurement

Saliva was obtained using a Sorbette sampling device (Salimetrics, State College, PA), which consists of a

TABLE 1. *Score for Topical Corticosteroid Treatment (Ref. 10)*

Score (total 10)	0	1	2	3	4
Potency of preparation (0 to 4)	None	Mild	Strong	Very strong	Strongest
Body surface area treated, % (0 to 3)		<9		>36	
Treatment duration* (0 to 3)		<7	≥ 7	Continuous	

*Days in the past month.

TABLE 2. *Clinical Characteristics*

Characteristic	Mild n = 12	Moderate n = 14	Severe n = 12	p-value*
Sex, male/female, n	6/6	9/5	9/3	0.44
Age, months, median (range)	19 (4–66)	21 (3–60)	10.5 (3–57)	0.41
SCORAD, median (range)	16 (8–25)	40 (26–48)	64.5 (51–86)	<0.001
Score for TCS treatment, median (range)	4.5 (4–7)	5 (0–7)	0 (0–7)	0.38
Non-TCS users, n	0	2	7	0.002
Number of venipunctures, median (range)	2 (1–3)	1 (1–4)	1 (1–3)	0.10
Total immunoglobulin E, IU/mL, median (range)	163 (5.2–4,562)	212 (12.9–9,500)	1,093.5 (55.6–4,161)	0.12

TCS, topical corticosteroid.
*Kruskal–Wallis test or chi-square test.

microsponge with a short plastic shaft as a handle. After the microsponge had been put under the tongue for 1 minute, the Sorbette was placed in a plastic tube and centrifuged for 15 minutes at 1,800 *g*, resulting in a clear, watery supernatant. The samples were stored at −30°C until analysis. For cortisol determination, 50 μL of saliva was used for duplicate analysis with a salivary cortisol enzyme-linked immunosorbent assay kit (Salimetrics), according to the manufacturer's protocol.

Statistical Analysis

Salivary cortisol concentrations did not show a normal distribution and were therefore log-transformed for analysis. Group variables were compared using the Mann–Whitney *U* test, the Kruskal–Wallis test, or the chi-square test. The paired *t* test was used to compare salivary cortisol response to the stressor (venipuncture). Spearman rank correlation was used to investigate the relationship between the parameters of the salivary cortisol response to the stressor and the variables (disease severity and TCS treatment). Data were analyzed using STATA software (Windows version 8.0, Stata Corp., College Station, TX). $P < 0.05$ was considered to indicate statistical significance in all comparisons.

RESULTS

Clinical Characteristics

Clinical characteristics of the patients are summarized in Table 2. The patients were grouped according to severity of AD (mild [12 patients], moderate [14 patients], and severe [12 patients]). There were no significant differences between the AD severity groups in terms of the age, sex, score for TCS treatment, number of venipunctures, or total immunoglobulin (Ig)E level (IU/mL), but there was a statistically significant difference in the number of patients not treated with TCS ($p = 0.002$).

Salivary Cortisol Response to Venipuncture

First, we evaluated whether venipuncture could be a stressor that induced significantly higher salivary cortisol levels in young children with AD. The number of venipunctures and the change in salivary cortisol level were investigated for possible association in patients in whom—for technical reasons—several venipunctures were needed to achieve blood sampling. The change in salivary cortisol level was calculated by dividing the postvenipuncture cortisol concentration by the prevenipuncture cortisol concentration, yielding a ratio. When subjects were stratified according to number of venipunctures, the increase in salivary cortisol level depended on the number of venipunctures ($p = 0.04$; Kruskal–Wallis test, Fig. 1). This result suggested that venipuncture was a sufficient stressor to induce a change in cortisol level in children younger than 6 years of age.

There were no significant differences in prevenipuncture and postvenipuncture salivary cortisol levels between the three groups, but the salivary cortisol level increased significantly after venipuncture in all groups ($p < 0.05$; paired *t* test, Fig. 2).

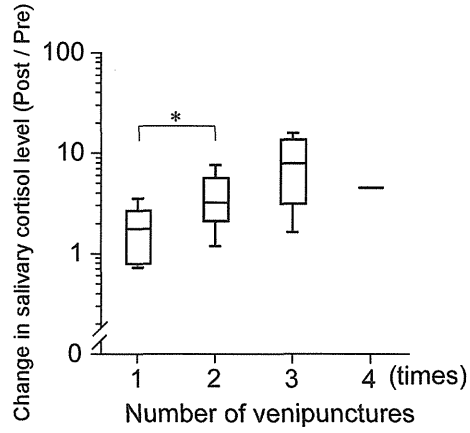

Figure 1. Change in salivary cortisol level (post/pre) versus number of venipunctures (*p = 0.02; Mann–Whitney test).

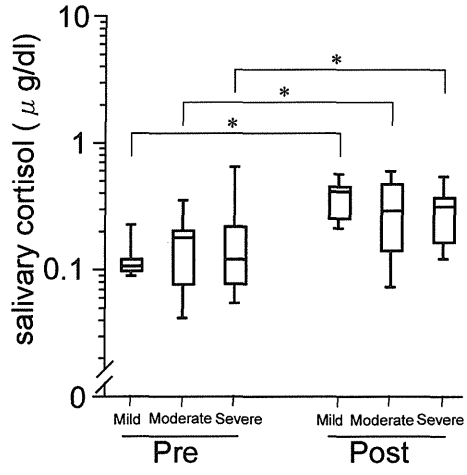

Figure 2. Salivary cortisol level in response to venipuncture in individuals with atopic dermatitis (*p < 0.05; Paired *t* test).

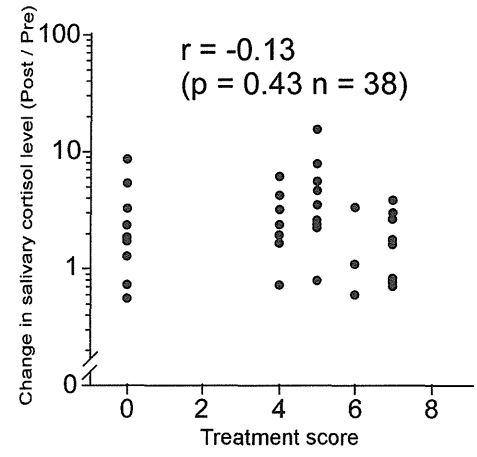

Figure 4. Correlation between change in salivary cortisol level (post/pre) and treatment score ($n = 38$, $r = -0.13$, p = 0.43).

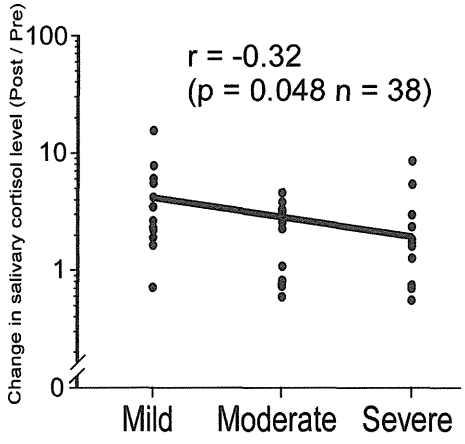

Figure 3. Correlation between change in salivary cortisol level (post/pre) and severity of atopic dermatitis ($n = 38$, $r = -0.32$, p = 0.048).

Correlation Between Salivary Cortisol Response and Severity of AD or TCS Treatment

The change in salivary cortisol level correlated significantly with the pre- and postvenipuncture salivary cortisol levels ($r = -0.50$, p = 0.01; $r = 0.61$, p < 0.001, respectively; data not shown). Conversely, it correlated negatively with severity of AD ($r = -0.32$, p = 0.048; Fig. 3). No significant correlation was found between change in salivary cortisol level and score for TCS treatment (p = 0.43; Fig. 4), age, sex, or total IgE (data not shown).

DISCUSSION

In previous studies, children and adults with AD showed attenuated salivary cortisol responses to psychosocial stressors, suggesting hyporesponsiveness of the HPA axis, which may be one explanation for stress-induced exacerbation of AD symptoms (3,4), but few studies have been performed in young children, who are vulnerable to systemic side effects of TCS therapy. In this study, we found that salivary cortisol response to the stress of venipuncture in young children with AD correlated negatively with the severity of symptoms.

We first evaluated whether venipuncture could be a stressor in children younger than 6 years of age. Although previous studies reported that changes in cortisol level after venipuncture were not significant in children aged 6 and older (19,20), we found that venipuncture is a stressor in children younger than 6 years of age. This difference might be because children have more occasions to undergo venipuncture and become used to it as they get older. Although venipuncture is difficult to standardize, and the individual characteristics of the children would influence its role as a stressor, salivary cortisol level rose dependent on the number of venipunctures. These results suggest that venipuncture is a sufficient stressor to induce a change in cortisol level in children younger than 6 years of age. The number of venipunctures did not differ significantly between the three groups of patients with different AD severity (mild 1.75 ± 0.72, moderate 1.29 ± 0.80, severe 1.42 ± 0.64, mean ± SD; p = 0.10), suggesting that patients in all three groups received virtually the same level of stress.

The salivary cortisol response to venipuncture as a stressor correlated negatively with the severity of AD. This result is in line with previous studies that found a weaker cortisol response to stressors in adults and children with AD (3,4). Meanwhile, there were no significant

differences in prevenipuncture cortisol levels between the groups with various AD severity, which is also in line with previous studies (3,4). Dysfunction of the HPA axis in individuals with AD may become apparent only when a stress stimulus is present.

According to a previous study (21), the normal salivary cortisol level in young children was approximately 0.16 to 0.36 μg/dL at baseline. The cortisol level in each group of subjects before venipuncture was compatible with that range, and there were no significant differences between the groups. Therefore, we think that our time points for performing venipuncture (10:00 A.M. and 3:00 P.M.) were sufficient for detecting any elevation in cortisol level, regardless of whether severe pruritus might have somewhat altered the circadian rhythm of cortisol release.

The underlying psychobiologic mechanisms of hyporeactivity of the HPA axis in patients with AD are not fully understood. Historically, these changes have been interpreted as a consequence of an ongoing chronic allergic inflammatory process, which releases pro-inflammatory cytokines (22). Some studies reported that an atopic disposition in neonates is associated with greater responsiveness of the HPA axis to stressors, which may promote the development of AD in later life (21,23). It remains to be determined whether these changes in HPA function precede or follow the onset of AD.

Most individuals with AD have high serum concentrations of total and allergen-specific IgE, and the severity of AD is known to be weakly associated with serum IgE levels and degree of Th2-type immune predisposition (24). In the present study, we also found that serum IgE levels were higher in those with more severe AD, but the differences were not statistically significant, presumably because of limited power or the fact that we studied very young infants, but further study is needed to elucidate whether hyporeactivity of the HPA axis is simply a consequence of the chronic inflammatory process or is specific to Th2-type immune responses.

The effects of TCS on HPA function in children with AD have been studied using various methods but with contradictory results (8–10). Our present study found no significant correlation between salivary cortisol level and previous TCS treatment, suggesting that there are other factors related to the disease. Our results are in line with previous findings showing that patients with AD and not treated with TCS had a weaker cortisol response than control subjects (3,4). Moreover, some studies showed that a significant decrease in the disease activity of AD after intensive treatment with large amounts of a potent TCS during hospitalization was associated with normalization of the basal serum cortisol level compared with levels at admission (5,11). These results suggest that the disease activity of AD, rather than TCS use, is responsible for dysfunction of the HPA axis in patients with severe AD.

In children with AD, percutaneous absorption of TCS was proven to be significantly lower in the convalescent phase of the disease than in the acute phase, probably because of the restoration of the skin barrier (25). Although percutaneous absorption of a potent TCS is likely to occur, especially during the acute phase of severe AD, the positive effect of adequate disease control seems to clinically outweigh the suppressive effect on adrenal gland function. Therefore, early restoration of the skin barrier by appropriate TCS therapy might contribute to reducing any undesirable effect of TCS on the HPA axis in individuals with AD in the long term. Good control of AD would improve HPA axis function, which might reduce stress-induced exacerbation of AD symptoms.

In addition, to clarify the effect of TCS on the HPA axis in patients with AD, we would like to compare the cortisol responses of patients with similar severity of AD but different TCS usage. We believe that appropriate use of TCS may improve the skin condition and quality of life of patients and in turn improve the HPA axis response. Suppression of the HPA axis can be seen only in patients with extremely severe AD or inappropriate administration of TCS. Further large-scale, longitudinal studies should be undertaken to elucidate the underlying mechanisms of HPA axis hyporeactivity in patients with AD. Limitations of this study are that the number of subjects was not sufficiently large and the sleep patterns of the patients were not closely determined, but a strength of the study is that we can evaluate HPA axis function to stress non-invasively in young children with AD.

Salivary cortisol responsiveness to the stress of venipuncture correlated negatively with the severity of AD but showed no correlation with previous use of TCS in young children with AD. These findings have major implications for daily practice when treating young patients with moderate to severe AD and steroid phobia.

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Anti-inflammatory effects of high-dose IgG on TNF-α-activated human coronary artery endothelial cells

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High-dose infusion of IgG (IVIG) is used to treat autoimmune and inflammatory diseases, including Kawasaki disease (KD). Although the immunomodulatory effects of IVIG on blood cells such as macrophages have been well studied, its effects on tissue cells remain unclear. Here, we show that high-dose IgG specifically and completely inhibited TNF-α-induced, but not IL-1β-induced, secretion of proinflammatory cytokines such as G-CSF and IL-6 by cultured human coronary artery endothelial cells (HCAECs). High-dose IgG did not inhibit TNF-α-mediated early signaling events of the NF-κB and MAPK pathways but it potently inhibited gene expression of G-CSF and IL-6 12 h after TNF-α-stimulation. Interestingly, suppression of the G-CSF and IL-6 gene expression correlated closely with functional inhibition of a transcription factor, C/EBPδ, whose binding sites in the promoters of *G-CSF* and *IL-6* have been shown to be critical for their transcriptional activation. Furthermore, the inhibitory effect of intact IgG on HCAECs was exerted mainly via its F(ab')$_2$ fragment, and not its Fc fragment. These findings suggest that the clinical effects of IVIG on KD patients are at least in part due to its direct anti-inflammatory effects on the coronary endothelium, which is a major lesion site in the pathogenesis of KD.

Keywords: Coronary artery endothelial cells · IVIG · Kawasaki disease · TNF-α

Supporting Information available online

Introduction

Intravenous infusion of IgG was originally used as a replacement therapy for patients with hypogammaglobulinemia in the early 1950s. High-dose infusion of IgG (IVIG) is now used to treat autoimmune and inflammatory diseases such as idiopathic thrombocytopenic purpura, Guillain-Barre syndrome, and Kawasaki disease (KD). To date, a number of possible mechanisms for the immunomodulatory and anti-inflammatory effects of IVIG therapy have been described [1, 2], including anti-complement effects [3], anti-idiotypic neutralization of pathogenic autoantibodies [4], immune regulation via an inhibitory Fc receptor [5, 6], enhancement of regulatory T cells [7] and inhibition

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of Th17 differentiation [8]. Thus, IVIG can mediate a wide variety of biological and immunomodulatory effects via various types of blood cells. However, its effects on tissue cells remain unclear.

KD is an acute systemic vasculitis seen in infants and young children [9, 10], and it is frequently associated with coronary artery aneurysms [11]. IVIG is a well-established standard therapy for KD that effectively reduces systemic inflammation and the incidence of coronary artery lesions (CALs) [12–14]. The clinical evidence strongly suggests that IVIG exerts its beneficial effects by attenuating coronary artery inflammation. However, the mechanisms underlying these clinical effects of IVIG on coronary endothelium are not well understood, and some patients do not respond to IVIG and develop CALs. Thus, we examined the in vitro effects of high-dose IgG on cultured human coronary artery endothelial cells (HCAECs), which is a major lesion site in the pathogenesis of KD.

We used TNF-α as an inflammatory stimulus in most of our in vitro experiments for the following reasons. First, during the acute phase of KD, serum levels of TNF-α are significantly elevated and correlate with the incidence of CALs in acute KD patients [15, 16]. Second, TNF-α was shown to be necessary for induction of coronary artery inflammation and aneurysm formation in a murine model of KD [17]. Indeed, TNF-α blocking agents were clinically effective in patients with KD refractory to standard treatment with IVIG and aspirin [18], suggesting that TNF-α may be required for the development of CALs in clinical KD.

Results

Changes in gene expression in HCAECs after high-dose IgG treatment

We first performed gene expression profiling of HCAECs to examine the effects of high-dose IgG on TNF-α-inducible genes. The concentration of IgG employed in this study was usually 20 mg/mL. That is approximately equivalent to the blood IgG level after current standard IVIG therapy for KD patients (2 g/kg). HCAECs were cultured overnight in the presence and absence of TNF-α prior to addition of IgG. The cells were further cultured for 1, 3, 6, and 24 h after IgG addition, and the changes in gene expression were compared between unstimulated cells and the cells stimulated with TNF-α alone or with both TNF-α and IgG. Twenty-four hours after IgG addition, 201 probes were over or underrepresented in the TNF-α-stimulated cells by more than twofold compared with the unstimulated cells and the cells stimulated with both TNF-α and IgG (Supporting Information Fig. 1). Table 1 lists the top 10 probes that showed the greatest decrease at 24 h after IgG addition. Interestingly, such cytokine and chemokine genes as G-CSF, IL-1β, CCL8/MCP-2, and IL-6, which are known to be expressed in systemic inflammatory diseases, held the top five places.

Inhibitory effects of high-dose IgG on TNF-α, but not IL-1β-induced cytokines in HCAECs

We next attempted to confirm the microarray data and to determine the precise kinetics of TNF inducible cytokine genes by quantitative real-time PCR (qPCR). Induction of G-CSF mRNA began after 12 h and continued to increase until 48 h after TNF-α stimulation (Fig. 1A). Importantly, expression of G-CSF mRNA was completely blocked by simultaneous treatment with IgG but not dexamethasone, resulting in profound inhibition of G-CSF release into the culture supernatants after stimulation with both TNF-α and IgG (Fig. 1B). As shown in Fig. 1C, IL-6 mRNA was transiently expressed during the first few hours after TNF-α stimulation and returned to its basal level after 8 h. IL-6 mRNA was then reinduced after 12 h and continued to increase until 48 h after stimulation with TNF-α. Although high-dose IgG had no effect on the early transient induction of IL-6 mRNA, it completely suppressed the reinduction of IL-6 mRNA after 12 h. IgG also completely suppressed TNF-α-induced IL-1β mRNA expression, with similar kinetics as G-CSF (Fig. 1E). However, IL-1β protein was not detected in the culture supernatants after TNF-α treatment (data not shown), probably due to a defect in the caspase-1-dependent process that causes release of mature IL-1β [19]. Instead, a proform of IL-1β protein (31 kDa) was detected in HCAECs lysates by western blot analysis after 24 h of TNF-α stimulation, and its expression was completely inhibited by simultaneous treatment with high-dose IgG (Fig. 1F), in agreement with the results for mRNA expression (Fig. 1E). Thus, high-dose IgG completely inhibited TNF-α-induced expression of the proinflammatory cytokines later than 12 h after stimulation.

On the contrary, high-dose IgG did not suppress induction of such cytokine genes as G-CSF, IL-6, and IL-1β after IL-1β stimulation (Fig. 1G–L). Furthermore, the inhibitory effects of IgG on TNF-α-induced cytokines were dose dependent (Supporting Information Fig. 2), and pretreatment of HCAECs with IgG also completely inhibited G-CSF, IL-6, and IL-1β mRNA expression later than 12 h after TNF-α stimulation (Supporting Information Fig. 3).

Effects of high-dose IgG on TNF-α-induced activation of NF-κB and MAPK in HCAECs

We next examined the effects of IgG on the signaling cascades in the early response of HCAECs to TNF-α. In order to observe the effects of high-dose IgG on the TNF-α-induced early signaling events, HCAECs were pretreated with 20 mg/mL IgG overnight prior to TNF-α stimulation. As has been well demonstrated, NF-κB p65 was rapidly phosphorylated at 15 min after stimulation with TNF-α and its phosphorylation level remained higher than the basal level until at least 24 h (Fig. 2A). IκBα protein disappeared rapidly at 15 min after stimulation but then returned to the basal level at 3 h after stimulation. Importantly, pretreatment with IgG had no effect on either the NF-κB p65 phosphorylation or the IκBα degradation. We confirmed these findings by

Table 1. The top-10 genes downregulated in HCAECs by high-dose IgG treatment

Gene symbol	Fold change TNF/TNF+IgG	Probe ID	Description
CSF3/G-CSF	27.1	207442_at	Colony stimulating factor 3 (granulocyte)
IL1B	11.4	205067_at	Interleukin 1, beta
CCL8	11.2	214038_at	Chemokine (C-C motif) ligand 8
IL6	9.6	205207_at	Interleukin 6 (interferon, beta2)
IL1B	9.0	39402_at	Interleukin 1, beta
TNFAIP6	8.2	206026_s_at	Tumor necrosis factor, alpha-induced protein 6
TNFAIP6	7.4	206025_s_at	Tumor necrosis factor, alpha-induced protein 6
C3	7.0	217767_at	Complement component 3
ITGB8	6.2	226189_at	Integrin, beta 8
C15orf48	5.7	223484_at	Chromosome 15 open reading frame 48

EMSA and found that pretreatment with IgG did not suppress NF-κB activity (Fig. 2B). Also, pretreatment of HCAECs with IgG had no effect on phosphorylation of Erk or p38 MAPK (Fig. 2A). These results clearly indicate that high-dose IgG exerts no effect on TNF-α-mediated early signaling events, including NF-κB activation.

Rapid inhibition of cytokine gene expression by high-dose IgG

We next investigated the inhibitory effect of IgG at various time points after TNF-α stimulation. For this purpose, as shown in Fig. 3, HCAECs were stimulated with TNF-α alone (blue), and then 20 mg/mL IgG was added to the culture 8 (red), 12 (green) and 24 h (orange) later. IgG added at 8 and 12 h after TNF-α stimulation completely inhibited G-CSF, IL-6, and IL-1β mRNA expression until at least 72 h of stimulation (Fig. 3A, C, and E). In consequence, the protein levels of G-CSF and IL-6 in the culture supernatants were quite low even at 72 h after stimulation (Fig. 3B and D). Even when added after 24 h of TNF-α stimulation, IgG rapidly suppressed expression of mRNA of G-CSF, IL-6 and IL-1β to their basal levels at 12 h after IgG addition (36 h after TNF-α stimulation, Fig. 3A, C, and E). As a result, the protein levels of G-CSF and IL-6 at 48 and 72 h of TNF-α stimulation with IgG treatment

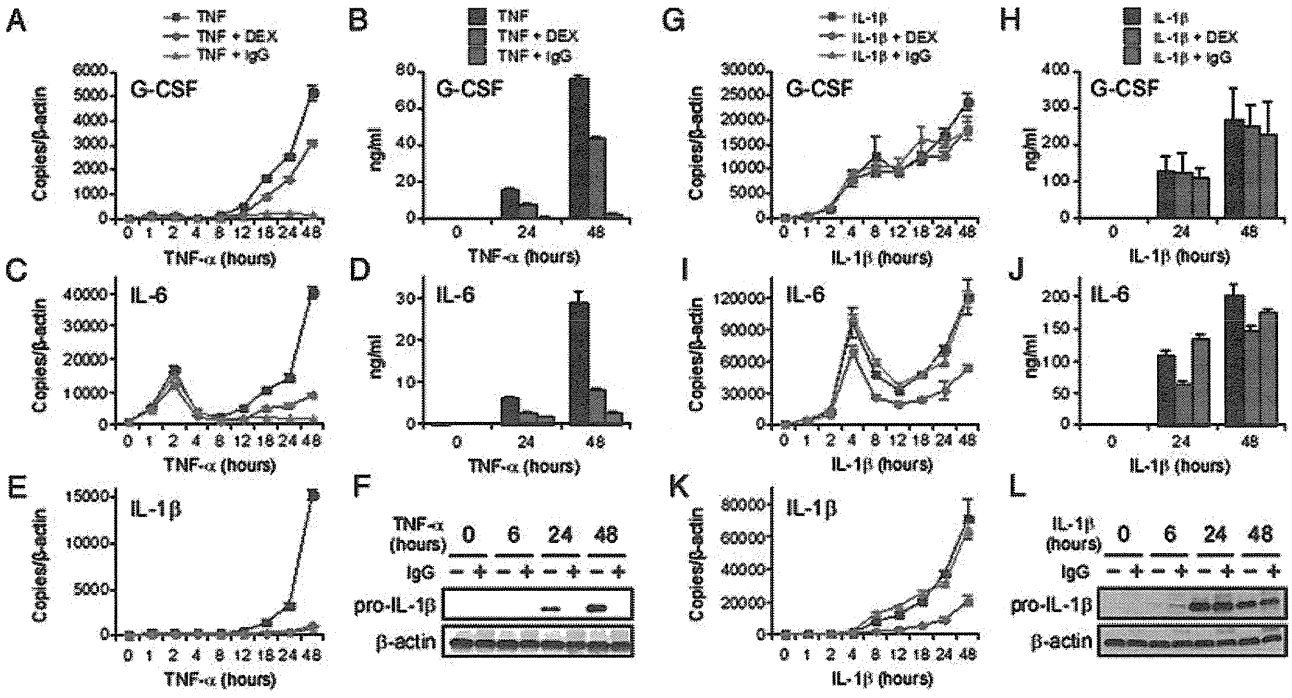

Figure 1. Inhibitory effects of high-dose IgG on TNF-α- and IL-1β-induced cytokines in HCAECs. HCAECs were treated with (A–F) 10 ng/mL TNF-α or (G–L) 10 ng/mL IL-1β in the presence and absence of 100 nM dexamethasone or 20 mg/mL IgG for the indicated time periods. The levels of mRNA for (A, G) G-CSF, (C, I) IL-6 and (E, K) IL-1β were examined by qPCR. Protein concentrations of (B, H) G-CSF and (D, J) IL-6 in the culture supernatants were measured by ELISA. (F and L) Whole-cell lysates from the indicated time points after treatment were subjected to western blot analysis for the expression of pro-IL-1β and β-actin (as a loading control). Data are shown as the mean ± SD of triplicate samples (A–E and G–J) and are representative of three experiments performed. Data are shown in (F, L) are representative of two experiments.

