Original article

Serum OX40 ligand: a potential marker of atopic dermatitis disease severity in children

Background: *OX40 ligand (OX40L) and OX40 are members of the tumor necrosis factor (TNF) and TNF receptor (TNFR) super families respectively. Recent studies have indicated the critical involvement of OX40/OX40L interaction in the pathogenesis of atopic dermatitis. To our knowledge, no data could be cited in literature concerning OX40L levels in serum or in other biological fluids of atopic dermatitis children.*

Objective: *This study was done to explore the expression of OX40L in the serum of atopic dermatitis children with respect to disease activity and severity.*

Methods: *This follow-up, case-control longitudinal study was conducted on 64 children as a stratified non-random sample; 34 with atopic dermatitis and 30 healthy children. Serum concentrations of OX40L were measured by sandwich enzyme immunoassay. The severity of atopic dermatitis was assessed according to the Leicester Sign Score (LSS), Simple Scoring System (SSS), Scoring Atopic Dermatitis (SCORAD) index, and Objective SCORAD.*

Results: *Serum OX40L levels (pg/ml) in atopic dermatitis patients were significantly elevated as compared to controls (176.6 ± 45.9) whether during flare (1007 ± 241.5) or quiescence (699 ± 198.5). There were significant positive correlations between serum OX40L levels and each of the LSS, SSS and SCORAD indices of atopic dermatitis disease severity, while it was insignificant regarding the objective SCORAD. However, when atopic dermatitis children were classified according to the objective SCORAD index of severity into mild, moderate and severe, it was found that the mean serum level in the severe group was significantly higher than the corresponding values of the mild or the moderate group. OX40L levels did not correlate with serum total IgE or absolute eosinophils count. Serum total LDH levels correlated positively with each of the serum OX40L levels and the LSS and SCORAD indices of severity.*

Conclusions: *Serum OX40L level is an objective reliable marker of atopic dermatitis severity in children. It may be useful for follow up and may help to improve research and management of this disease. Blockade of interactions between OX40 on Th2 cells and OX40L on activated dendritic cells using an OX40L-specific monoclonal antibody could represent a novel strategy for the treatment of atopic dermatitis.*

Keywords: Atopic dermatitis, LSS, OX40, OX40L, SCORAD, SSS, TNF.

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INTRODUCTION

The tumor necrosis factor receptor (TNFR) family consists of a number of type I transmembrane glycoproteins characterized by homologous cysteine-rich domains in their extracellular region. The intracellular parts of these proteins vary in size and structure, corresponding to the wide array of functions of TNFR proteins, ranging from regulation of cell activation and differentiation to induction of cell death. Twenty-two TNFR family members have been identified, including TNFR1, TNFR2, the low-affinity nerve growth factor receptor, CD27, CD30, CD40, CD95 (Fas), and CD134 (OX40). Except for the ligands of the low-affinity nerve growth factor receptor, the ligands of TNFR proteins form the family of TNF-related proteins. Most TNF family members can be expressed as type II transmembrane receptors, and some of these proteins mediate signal transduction via their intracellular domain. Several members of the TNFR family (e.g., CD95, TNFR1, and TNFR2) and the TNF family (e.g., TNF-α, CD40 ligand, and CD95 ligand) can be produced as

functional soluble proteins, as a consequence of either alternative mRNA splicing or proteolytic cleavage of the transmembrane protein[1].

OX40 ligand (OX40L) and OX40 are members of the TNF and TNFR super families respectively. OX40L (TNFSF4) was originally termed glycoprotein 34 kDa (GP34) whose expression on T cells was induced by the tax gene of human T cell leukemia virus-I-infected T cells. OX40L is a type II transmembrane glycoprotein that exists as a homotrimer. Human OX40L cDNA encodes a 183 amino acid (AA) polypeptide with an N-terminal cytoplasmic domain (AA 1-23) and a C-terminal extracellular domain (AA 51-183). OX40L may be shed from the membrane and exist in a soluble form[2].

OX40 (CD134/TNFRSF4) is primarily known to be a T cell activation marker that is preferentially expressed by activated $CD4^+$T cells, whereas OX40L is mainly expressed by antigen presenting cells, including activated dendritic cells, B cells, macrophages, and Langerhans cells, as well as by T cells and endothelial cells. Unlike another costimulatory molecule, CD28, which plays an important role in T cell priming, OX40-OX40L interactions have been shown to be crucial for T cell activation and survival and for the generation of memory T cells from activated effector T cells[1].

OX40L/OX40 interaction is important for mediating both Th1 and Th2 responses. A recent study has made a clear distinction in requirements for OX40L between the 2 types of responses. They proposed a determinant role for OX40L in promoting Th2 polarization and response of naive CD4+ T cells in the absence of IL-12, while in the presence of IL-12, OX40L served to increase Th1 responses. OX40L-mediated polarization of T cells along the Th2 lineage was initiated by dendritic cell activated with the cytokine thymic stromal lymphopoietin (TSLP)[3].

Recent studies have indicated the critical involvement of OX40/OX40L interaction in the pathogenesis of a variety of immunologic abnormalities of inflammatory, autoimmune, infectious, allergic, and allotransplantation-related diseases. In particular, OX40L on vascular endothelial cells may play a role in inflammatory vasculitis. Blockade of OX40/OX40L interaction has been shown to prevent, cure, or ameliorate these diseases. In contrast, activation of OX40 is known to break an existing state of tolerance in malignancies, leading to a reactivation of antitumor immunity[4].

Recent advances in understanding the cellular and molecular mechanisms of atopy have shed light on potential targets for the development of new therapies for atopic diseases. Recent analyses of microarray experiments on TSLP-stimulated dendritic cells have shown that OX40L is strongly induced by TSLP. In vitro neutralization of OX40L activity was able to block TSLP-activated dendritic cells polarization of naive T cells[5]. Blockade of interactions between OX40 on Th2 cells and OX40L on TSLP-activated dendritic cells using an OX40L-specific monoclonal antibody, had inhibited Th2 cell-mediated immune responses in both mouse and nonhuman primate models of allergic inflammation. The results point to potential therapeutic approaches to targeting the cellular and molecular mechanism underlying OX40L-mediated allergic inflammation.

The aforementioned data suggest that OX40/OX40L interaction probably play a significant role in T cell responses and immune regulation of various T cell mediated disorders. To our knowledge, no data could be cited in literature concerning OX40L levels in serum or in other biological fluid of atopic dermatitis children. Therefore, this case-controlled longitudinal study was done to explore the expression of OX40L in serum of atopic dermatitis children and to identify the clinical relevance of measuring serum OX40L to disease activity and severity.

METHODS

This follow-up, case-control study was conducted on 64 children as a stratified non-random sample; 34 with atopic dermatitis and 30 healthy children, during the period from the first of February 2008 to the end of November 2008. All children were enrolled from the Pediatric Allergy and Immunology Unit, and Pediatric and Dermatology outpatient clinics of Ain Shams University Hospitals, Cairo. An informed consent was obtained from the parents or caregivers of each child before enrollment.

Patients: Children with atopic dermatitis were diagnosed according to the criteria proposed by Hanifin and coworkers[6]. They were subjected to a detailed history taking and thorough clinical examination to exclude subjects with any concomitant diseases. The parents were subjected to a questionnaire concerning the precipitating factor(s). A possible cause was traced in 25 children being exposed to food allergens: egg (8), milk (7), banana (3), fish (3), and multiple food allergens (4). In 9 children, the cause was not clear.

•Assessment of the clinical severity of atopic dermatitis: For convenience, the severity was assessed according to four different scoring systems; the Leicester Sign Score (LSS), Simple scoring system of Costa (SSS), Scoring Atopic Dermatitis (SCORAD) index, and Objective SCORAD.

- The severity in LSS is scored by 6 clinical features (erythema, purulence, excoriation/crusting, dryness/scaling, cracking/fissuring, and lichenification) graded at 6 defined body sites on a scale of 0 (none) to 3 (severe) [LSS score range: 0 to 108][7].
- The SSS or Costa's score calculates the intensity of 10 signs and symptoms along with involvement of 10 different skin regions. The intensity criteria are: erythema, edema, vesicles, crusts, excoriation, scales, lichenification, pigmentation or depigmentation, pruritus and loss of sleep[8].
- The SCORAD index was used to evaluate the severity of eczema by combining 2 objective symptoms (extent and intensity of eczema) and 2 subjective symptoms (pruritus and sleep loss) to a maximum score of 103 points[9].
- The Objective SCORAD is a modification of the SCORAD that excludes the subjective symptoms as pruritus and sleep loss, to minimize the errors caused by variability in patient's ages and backgrounds. A proposal for severity grading of atopic dermatitis by using only objective criteria is as follows: (mild atopic dermatitis: score < 15; moderate atopic dermatitis: score = 15-40, and severe atopic dermatitis: score >40)[10].

Controls: Thirty clinically healthy children with no history of atopy were included as a control group. They were subjected to general and systemic clinical examination to exclude any current illness.

Exclusion criteria:

1. All subjects (patients and controls) with clinical or laboratory evidences of parasitic or other concomitant systemic or dermatological illnesses were excluded from the study.
2. Atopic dermatitis children were enrolled after exclusion of a past, current or family history of other atopic or allergic diseases such as atopic asthma, allergic rhinitis or allergic conjunctivitis.
3. Atopic dermatitis patients receiving systemic corticosteroids or immunosuppressive therapy were excluded. Topical corticosteroids and antihistamines were stopped for at least 2 weeks before inclusion in the study. Only topical emollients were used.
4. Control subjects were enrolled after exclusion of a past, current or family history of atopic or allergic diseases.
5. Control subjects with peripheral blood eosinophilia or elevated serum total IgE for age were excluded.

Study design

At enrollment, all children with atopic dermatitis were clinically evaluated for the severity while presenting with atopic dermatitis flare, then sampled for OX40L assay. Patients were followed up till quiescence of the disease by symptomatic therapy (systemic and topical corticosteroids, immunosuppressive therapy and antihistamines were stopped for at least 2 weeks before sampling), when follow up blood samples were collected. Quiescence and response to treatment was checked by using the Physician's Global Assessment (PGA) score. The PGA is an overall assessment from 0 to 5 of a patient's eczema, taking into consideration the quality and extent of lesions relative to the baseline assessment (0 = clear [100%], 1 = almost clear [90% to 99% improvement], 2 = marked improvement [50% to 89%], 3 = modest improvement [<50%], 4 = no change, and 5 = worse).

Laboratory evaluation

Blood sampling: Five ml of venous blood were collected under complete aseptic precautions and divided into 2 parts. Two ml were mixed with EDTA for blood counting. Sera were separated from the remaining three ml and were stored at -20°C for quantitative measurement of serum OX40L, total IgE and lactate dehydrogenase (LDH). Repeated thawing and freezing was avoided. Hemolyzed and lipemic samples were excluded.

Study measurements:

1-Complete blood count: The blood count was performed in every subject with the Coulter counter *(Coulter MicroDiff 18, Fullerton CA, USA)*. The differential leukocytic counts were estimated manually form the blood film and expressed in absolute count values. Infants and children whose absolute eosinophils counts (AEC) exceeded the normal reference values for age were considered to have peripheral blood eosinophilia[11]. Blood sampling of all subjects was performed at the same time (10 am) to avoid diurnal variations in eosinophils counts.

2-Serum total IgE: Serum total IgE was assayed in all subjects by quantitative enzyme immunoassay *(BioCheck, Inc 323 Vintage Park Dr. Foster City, CA 94404)*. Results were expressed in IU/ml.

Owing to the variability of serum total IgE levels with age; we calculated the percentage of the subject's actual level to the highest normal for age[11]. IgE levels used in the correlations were both the measured and the calculated percentage values. The serum total IgE level that exceeded the highest normal for age was considered elevated.

3-Serum total lactate dehydrogenase (LDH) assay: Serum total LDH (U/L) was measured in the atopic dermatitis group only during flare and in the healthy subjects. LDH assay was performed on an automatic chemistry analyzer (Synchron CX5 system, Beckman, Inc., Fullerton, California, USA).

4-Stool and urine analysis: To exclude any parasitic infestations when suspected.

5-Quantitative determination of serum OX40 Ligand: The Quantikine OX40 Ligand immunoassay is a 4.5 hour solid-phase ELISA. Reagents were supplied by *Quantikine® (R&D Systems, Inc. 614 McKinley Place N.E. Minneapolis, MN 55413 USA) (Catalog Number DOXL00).*

Statistical Analyses

All statistical analyses of the data were carried out using SPSS (Statistical Package for the Social Science) version 11 for Windows system. Data were statistically represented in terms of range and mean ± standard deviation (SD). As regards parametric data, Student's "t" test was used to compare between 2 groups, and ANOVA test (F-value) to compare more than 2 groups. The relation between various numerical parameters was studied by the Pearson correlation coefficient (r) test with graphic representation using linear regression line; r value was considered weak if <0.5, moderate if ranged between 0.5-0.75 and strong if >0.75. P value less than 0.05 was considered significant.

RESULTS

According to the SCORAD index of severity, 14 (41%) of the studied patients had moderate atopic dermatitis and the remaining 20 patients (59%) had severe atopic dermatitis. With respect to the objective SCORAD index, 7 (20.5%) had mild atopic dermatitis, 13 (38.5%) had moderate atopic dermatitis and 14 (41%) had severe atopic dermatitis. When atopic dermatitis patients and controls were compared with respect to serum total LDH, serum total IgE levels and AEC, statistically significant higher values were detected in the former group ($p < 0.05$ for all) (table 1).

Serum OX40L levels during atopic dermatitis flares ranged between 443 and 2041 pg/ml (mean ± SD = 1007 ± 241.5 pg/ml). After subsidence of acute attacks, there was a significant decrease in OX40L serum levels (range = 421-980 pg/ml; mean ± SD = 699 ± 198.5 pg/ml ($t = 0.78$; $p < 0.001$). The healthy children had much lower serum levels (range = 37-242; mean ± SD = 176.6 ± 45.9 pg/ml) as compared to the patients' data whether during flare ($t = 1.3$; $p < 0.0001$) or quiescence ($t = 0.9$; $p < 0.0001$) (figure 1).

There was no significant difference in the OX40L serum levels between male and female subgroups in atopic dermatitis patients and controls ($p>0.05$). Serum OX40L levels could not be correlated to age both in the patient and the control groups.

As regards the indices of atopic dermatitis severity, there were significant positive correlations between serum OX40L levels and each of the LSS, SSS and SCORAD indices of severity (figure 2), while it was insignificant regarding the objective SCORAD. According to the objective SCORAD index of severity, we found that the mean serum OX40L level of the severe atopic dermatitis group and that of the moderate group were significantly higher than the corresponding values of the mild group. Only mild cases had statistically comparable values to those of controls (figure 3).

There were no significant correlations between serum OX40L levels or the indices of atopic dermatitis severity and serum total IgE level or AEC in patients group. Serum total LDH levels correlated positively with each of the serum OX40L levels ($r = 0.63$; $p<0.01$) and the LSS ($r = 0.43$; $p<0.05$) and SCORAD ($r = 0.54$; $p<0.05$) indices of severity.

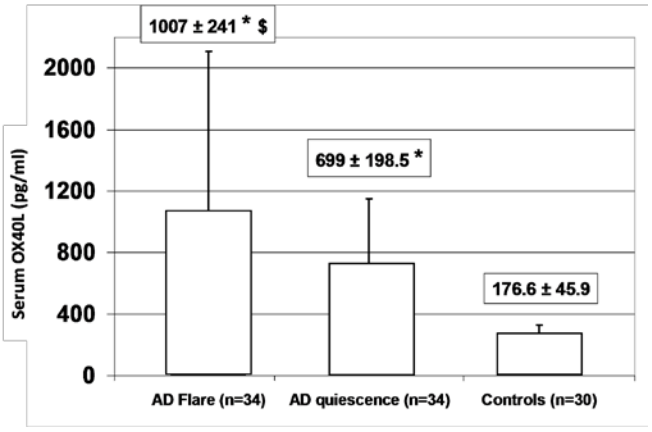

Figure 1. Serum OX40L levels (pg/ml) in atopic dermatitis children during flare and quiescence in comparison to controls.

*: Statistical significant difference as compared to controls ($p < 0.0001$)

$: Statistical significant difference (flare versus quiescence; $p < 0.001$)

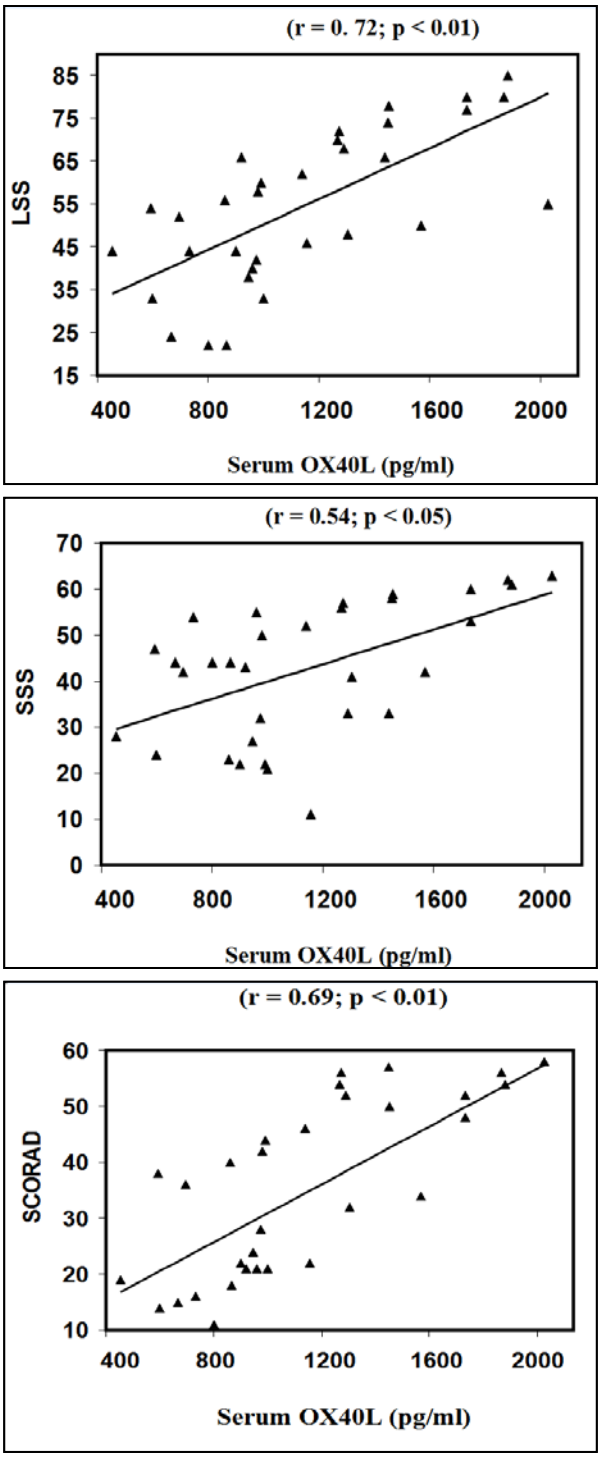

Figure 2. Significant positive correlations between serum OX40L levels (pg/ml) and the Leicester Sign Score (LSS), Simple scoring system of Costa (SSS), and the SCORAD index of atopic dermatitis disease severity.

Table 1. Demographic, clinical, and laboratory data of patients with atopic dermatitis during flare and of controls. Values are given as range and mean (SD)

Variable	Atopic Dermatitis (n = 34)	Controls (n = 30)
Age (years)	2-11 6.95 (1.92)	3-14 7.23 (2.41)
Sex (M:F)	20:14	18:12
Leicester Sign Score (LSS)	20-85 54.82 (10.62)	---
Simple Scoring System (SSS)	10-63 47.11 (8.89)	---
Scoring Atopic Dermatitis (SCORAD)	13-58 36.15 (5.67)	---
Objective SCORAD	9-45 24.89 (7.52)	---
Serum total LDH (U/L)	110-1890 985 (314.32)*	40-150 80 (17.58)
Absolute eosinophil Count (10^9/L)	200-950 596 (197)*	20-110 55(10.86)
Serum total IgE (IU/mL)	15-390 199 (86.73)*	3-49 18 (7.41)

* Significant as compared to controls

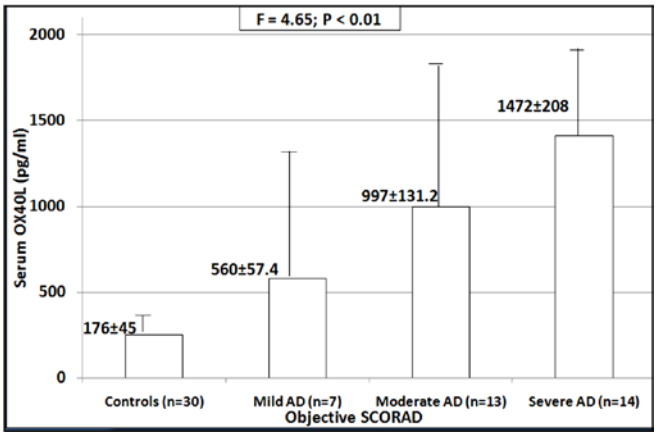

Figure 3. Serum OX40L levels (pg/ml) in atopic dermatitis patients according to the objective SCORAD index of severity.

DISCUSSION

The clinical assessment of atopic dermatitis is complicated by variability among investigators; therefore an objective laboratory marker might have a more precise basis for monitoring disease activity and severity, and for evaluating the success of treatment[12].

In this study, serum OX40L levels of atopic dermatitis patients were significantly elevated as compared to control subjects whether during flare or quiescence. This result probably reflects the up regulation of OX40L in atopic dermatitis, presenting a potentially useful marker for the presence of an atopic reaction. The significant drop in serum levels during quiescence not only signifies the potentiality of OX40L as an efficient marker of disease activity, but signifies also the importance of up regulation of OX40L by allergen exposure and microbial products in the initiation and amplification of the atopic skin response.

The role of OX40/OX40L interaction during T-cell-dendritic cell interaction in atopic dermatitis is to be elucidated. OX40L might represent an early trigger of the allergic immune cascade. Unlike another costimulatory molecule, CD28, which plays an important role in T cell priming, OX40/OX40L interactions during T-cell-dendritic cell interaction have been shown to be crucial for clonal expansion of antigen-specific T-cells, the generation, activation, and survival of T-cell and for the generation of memory T cells from activated effector T cells[3].

A large variety of laboratory measurements have been linked to disease activity and severity in atopic dermatitis, however most of them lack specificity [(13)]. This study demonstrated that OX40L concentrations correlated positively with the indices of atopic dermatitis severity namely; LSS, SSS and SCORAD.

The objective SCORAD index differentiated children with mild disease from those with moderate-to-severe disease, so that serum OX40L levels in subjects categorized as severe atopic dermatitis were significantly higher as compared to the moderate and mild groups. This substantiates the usefulness of OX40L as an objective marker of disease severity in atopic dermatitis.

In the current study, there were no significant correlation between serum OX40L levels or the clinical indices of atopic dermatitis severity and serum total IgE levels in patients group. Laske and Niqqemann[14] reported that serum IgE levels correlated with the degree of atopic dermatitis. Dhar et al.[15] reported that IgE levels and absolute eosinophilic count were significantly higher in atopic dermatitis patients than in controls (as in the current study), however, IgE levels and the absolute eosinophilic count showed significant covariance with disease severity. They revealed that there was a non-homogeneous distribution of both IgE levels and absolute eosinophilic count which were reflected in the large range and higher standard deviation. They also found a significant association of these two parameters with a family history of atopic dermatitis only when both parents were affected. Also, they found a significant association of these two parameters with a history of bronchial asthma in patients with atopic dermatitis but not with allergic rhinitis.

IgE levels are known to fluctuate throughout the disease stages. In this study, atopic dermatitis subjects were recruited at different stages of disease activity. Noteworthy, atopic dermatitis is not exclusively an IgE-mediated allergic disorder; where a fraction of atopic dermatitis patients had a normal or even decreased total IgE levels; these patients had a non-IgE associated type of the atopic eczema/dermatitis syndrome or the formerly called intrinsic type of atopic dermatitis. Although patients with atopic dermatitis frequently possess IgE antibody specific for inhalants or food allergens, it is not generally possible to induce skin lesions or atopic dermatitis by intradermal injection of the suspected allergen[16].

In the current study, there was no significant correlation between serum OX40L levels or the clinical indices of atopic dermatitis severity and absolute eosinophilic count. It is not clear whether OX40L may have a role in regulating the constitutive numbers of eosinophils in peripheral blood by inducing eosinophil chemotaxis. Moreover, the activity and the release of eosinophils were not always correlated with their count[17].

It was reported that the eosinophils counts roughly correlated with the disease severity, and that very high counts were common in severe cases of atopic dermatitis who had a personal or family history of respiratory atopy, while normal or moderately elevated counts were obtained in severe cases of 'pure' atopic dermatitis who had neither a personal nor a family history of respiratory atopy[18]. Thus, it was suggested that disease severity and personal or family history of respiratory atopy are important factors in determining high blood eosinophils counts in atopic dermatitis. Since children recruited in this study had 'pure' atopic dermatitis without any history of other atopic disorders, the lack of correlation between the indices of atopic dermatitis

severity and the absolute eosinophils count could be expected.

Therefore, it seems that atopic dermatitis severity scores and both IgE and eosinophils count represent different domains in atopic dermatitis assessment. They do not necessarily correlate well with each other and all three aspects must be individually evaluated to assess the well being of these patients.

Serum total LDH levels of atopic dermatitis patients during flare were significantly higher than control values. LDH levels correlated positively with each of the serum OX40L levels and the LSS and SCORAD indices of severity. LDH was previously reported as a useful marker of severity of atopic dermatitis disease[19]. Elevated levels can be attributed to its release from damaged lesional epidermal cells into peripheral blood reflecting the extent of skin involvement and the degree of epidermal injury.

In conclusion, the overexpression of OX40L in atopic dermatitis patients during flare and quiescence in comparison to healthy children signifies the importance of up regulation of OX40L by allergen exposure and microbial products in the initiation and amplification of the atopic skin response during T-cell-dendritic cell interactions presenting a potentially useful marker of the presence of an atopic response. The significant drop in serum levels during quiescence signifies the potentiality of OX40L as an efficient marker of disease activity. Moreover, the results of the current study namely the parallel association of OX40L with disease severity suggest a role for OX40L in the assessment of atopic dermatitis severity in children.

Our findings with respect to serum OX40L expression in relation to atopic dermatitis disease clinical and laboratory variables must be regarded as preliminary, pending longitudinal confirmation on a larger sample. Little is known about the functional involvement of OX40/OX40L interactions during T-cell-dendritic cell interaction in atopic dermatitis. Its role in the etiopathogenesis is far from clear yet, and should be clarified. Further study of OX40L functions within the TNF/TNFR super families individually and their interplay should provide insights into the pathogenesis of atopic dermatitis, and might have great implications for treatment of severe recalcitrant atopic dermatitis. Moreover, additional studies are recommended to clarify whether blocking OX40L or neutralizing its receptor by various neutralizing agents could represent a novel strategy for the treatment of atopic dermatitis.

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