Gap junction and atopic dermatitis: a study of connexin 43 mRNA expression in atopic skin lesions

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Background
Atopic dermatitis is a highly pruritic, chronic inflammatory skin disease. There is no doubt that there is an epidermal barrier defect in atopic dermatitis. Some recent studies have found a correlation between epidermal barrier function and tight junction. Recently a correlation between tight and gap junction was also reported.

Objective
To study the expression of connexin 43(Cx43) mRNA, an important constituent of gap junction in the skin in atopic dermatitis lesions and to compare it with healthy controls.

Patients and methods
The study was carried out in 20 patients with atopic dermatitis. Fifteen healthy volunteers served as controls. A 4-mm punch skin biopsy specimen was obtained from each patient and control for quantitative reverse transcription-PCR measurement of Cx43 mRNA expression. Biopsy specimens were stored at $-70°C$. Reverse transcription-PCR was conducted using the extracted RNA for the detection of the Cx43 gene.

Results
The mean Cx43 mRNA expression in the patients was $1.23 \pm 0.1\,\mu g/gm$ tissue and $0.27 \pm 0.05\,\mu g/gm$ in the control group. This denoted a significant statistical difference between the groups (P value = 0.000) revealing higher expression in patients with atopic dermatitis.

Conclusion
This study suggests a new hypothesis for atopic dermatitis pathogenesis showing a possible contribution of gap junction abnormality in the disease. These findings raise new future directions for management of patients with atopic dermatitis.

Keywords:
atopic dermatitis, connexin 43, gap junction, tight junction

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Introduction

Atopic dermatitis is a highly pruritic, chronic inflammatory skin disease that affects up to 20% of children worldwide and can persist into adulthood [1]. It has a significant impact on the quality of life of patients and their families and the economic impact is estimated to be in the billions of dollars [2]. More than 50% of patients develop asthma and other atopic disorders, further adding to the health and economic burden of this disease [3]. There is no doubt that there is an epidermal barrier defect in atopic dermatitis. The evidence to support this comes from the following findings: enhanced transepidermal water loss, reduced irritancy threshold, increased percutaneous absorption, and dry appearance of lesional skin [4]. The extent of barrier dysfunction correlates with the degree of inflammation within atopic dermatitis lesions and atopic dermatitis severity in general [5]. To maintain the structure and function of the epidermis a number of intercellular junctions exist, including tight junctions (TJ), gap junctions, adherens junctions, and desmosomes. These cell–cell contacts have multiple and diverse roles in regulating aspects of tissue adhesion, signaling, communication, differentiation, migration, proliferation, shape, permeability, polarity, and development [6].

Some recent studies have found a correlation between epidermal barrier function and TJ. They reported that claudin-1-deficient mice died within 24 h of birth with wrinkled skin, severe dehydration, and increased epidermal permeability as measured by dye permeability and transepidermal water loss. Importantly, these mice had a normal functioning stratum corneum (SC) (and normal expression of SC proteins) but a dysfunctional TJ [7]. Gap junctions (GJ) mediate direct communication between adjacent cells by means of the exchange of small molecules (< 1000 Da), including metabolites and second messengers. The protein subunits of GJ channels are called connexins (Cx). Six Cx form a hemichannel (connexon), and two connexons (one from each cell) form a GJ channel [8]. GJ have been shown to play important roles in the proliferation, migration, and differentiation of keratinocytes [9]. In humans more than 20 Cx are expressed and of these the Cx43 is abundantly expressed

in keratinocytes, dermal fibroblasts, vascular system, skin appendages, macrophages, neutrophils, and mast cells. It also plays a key role in cellular communication to maintain tissue homeostasis and angiogenesis [10]. However, no direct correlation has been reported between GJ and epidermal barrier function.

A correlation between TJ and GJ has been reported. Some studies have examined zonula occludens (ZO), ZO-1 and ZO-2 (considered as a TJ-associated protein), and reported zona occludens binding to Cx43 at different stages of the cell cycle. They found that ZO-1 bound preferentially with Cx43 in cells that were quiescent, suggesting that ZO-1 interaction with Cx43 may contribute to the stability of GJs [11,12]. Another study, using a Cx43-binding dominant-negative fragment of ZO-1, in osteoblast-derived cells, suggested a role for ZO-1 in regulating GJ assembly [13]. Consistent with the regulation of GJ formation, blocking ZO-1 binding to Cx43 has been reported to increase the size of GJs in HeLa cells [14,15]. Collectively, these studies suggest that ZO-1 binding to Cx43 acts to regulate GJ size and stability.

On the basis of the previous findings, we assumed that Cx43 expression may be affected in atopic dermatitis, whether being a primary or a secondary effect of the disease. Accordingly, we measured the level of Cx43 mRNA in 20 patients with atopic dermatitis in an attempt to clarify the relationship between GJ and this common skin disorder.

Patients and methods

The study was carried out in 20 patients with atopic dermatitis. They were selected from the Outpatient Clinic of the Dermatology Department, Faculty of Medicine, Cairo University. Written informed consent was obtained from each patient before initiation of the study. The study verified the protocol of the ethics committee of Kasr El Aini Hospital applied to human studies.

The patient group included 12 male and eight female. Their age ranged between 10–46 years. Patients with history of any cutaneous or systemic disease other than atopic dermatitis were excluded. Six patients in the studied group had a family history of atopy, whereas the rest were not aware of family members who had any manifestations of atopy. Six patients had a history of recurrent bronchial asthma. All patients stopped any systemic therapy for atopy 2 months before the start of the study.

The grade of clinical severity was evaluated according to Rajka and Langeland [16] mild (score: 3–4); moderate (score: 4.5–7.5); severe (score: 8–9).

According to the grading of disease severity (Table 1), patients with atopic dermatitis were divided into three groups:

(1) Mild cases (score: 3–4) included eight (40%) patients, four male and four female.

Table 1. Grading of disease severity in patients with atopic dermatitis

Patient	Extent[a]	Course[b]	Intensity[c]	Grade of severity (score)
1	1	2	1	Mild 4
2	2	1	3	Moderate 6
3	1	2	1	Mild 3
4	3	3	3	Severe 9
5	3	3	3	Severe 8
6	2	1	1	Mild 4
7	3	3	3	Severe 9
8	1	1	1	Mild 4
9	2	3	3	Severe 8
10	1	2	1	Mild 4
11	2	1	1	Mild 4
12	3	3	3	Severe 9
13	3	3	3	Severe 9
14	1	1	1	Mild 3
15	1	2	2	Moderate 5
16	1	1	2	Severe 8
17	1	1	1	Mild 3
18	2	2	2	Moderate 7
19	2	2	2	Moderate 6
20	2	2	1	Moderate 5

[a]Extent: the involved surface area of the body.
[b]Course: periods of remissions during a year.
[c]Intensity: patient experience of itch.

(2) Moderate cases (score: 4.5–7.5) included five (25%) patients, all of them were male.
(3) Severe cases (score: 8–9) included seven (35%) patients, three male and four female.

Controls

Fifteen age-matched and sex-matched healthy volunteers served as controls (six male and six female). Written informed consent was obtained from each control to participate in the study.

A 4-mm punch skin biopsy specimen was obtained from each patient and control for quantitative reverse transcription (RT)-PCR measurement of Cx43 mRNA expression. Biopsy specimens were stored at -70°C.

Detection of connexin 43 mRNA expression by reverse transcription-polymerase chain reaction
*RNA extraction*
Total RNA was extracted from skin tissue by the acid guanidinum thiocyanate-phenol-chloroform method. RNA content and purity were measured by a ultraviolet spectrophotometer.

*Reverse transcription-polymerase chain reaction experiments*
RT-PCR was carried out using the extracted RNA for detection of the Cx43 gene. For amplification of the target gene reverse transcription and PCR were run in two separate steps. Briefly, a reaction mixture of RT reaction containing 1 μg of total RNA, 0.5 μg of random primer, 5X RT buffer, 2.5 mmol/l of dNTP, 20 U RNase inhibitor, and 200 U Molney Murine Leukemic Virus RT in a total volume of 25 μl was incubated at 37°C for 60 min, and then heated to 95°C for 5 min to inactivate Molney Murine Leukemic Virus. PCR was carried out with 1.5 μl of RT products, 10X PCR buffer (without Mg^{2+}) 2.5 μl, 2.0 μl dNTP (2.5 mmol/l), 2.0 μl of $MgCl_2$

Table 2. The oligonucleotide primers sequence of studied genes

	Primer sequence	Annealing temperature (°C)	Product size (bp)
Connexin	Forward primer: 5′-GGAAGATTAGTTACAAATAGTTG-3′ Reverse primer: 5′-ATAGTTATCTATTGCATGAACTG-3′	65	310
β-actin	Forward primer: 5′-TGTTGTCCCTGTATGCCTCT-3′ Reverse primer: 5′-TAATGTCACGCACGATTTCC-3′	60	206
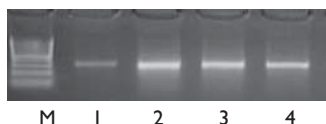
Figure 1.

An agarose gel electrophoresis show PCR product of connexin gene in different studied groups. Lane M: DNA marker with 100 bp. Lane 1: PCR products of connexin in control group. Lane 2, 3, and 4: PCR products of connexin in diseased group.

Figure 2.
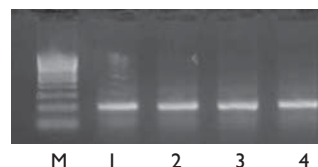
An agarose gel electrophoresis show PCR product of β-actin gene in different studied groups.

(25 mmol/l), 0.5 μl of each primer (20 μmol/l) of β-actin, 0.5 μl each primer of gene to be tested (20 μmol/l), and 1 U of Taq DNA polymerase (Promega Incorportion, Madison, USA), in a final volume of 25 μl. Thermal cycler conditions were as follows: a first denaturing cycle at 97°C for 5 min, followed by a variable number of cycles of amplification defined by denaturation at 96°C for 1.5 min, annealing for 1.5 min, and extension at 72°C for 3 min. A final extension cycle of 72°C for 15 min was included. The appropriate primer pairs, annealing temperature and product size is shown in Table 2.

Agarose gel electrophoresis
All PCR products were electrophoresed on 2% agarose stained with ethidium bromide and visualized by a ultraviolet transilluminator (Figs 1 and 2).

Semiquantitative determination of polymerase chain reaction products
Semiquantitation was performed using the gel documentation system (BioDO, Analyser, Biometra, Germany) supplied by Biometra according to the following amplification procedure: relative expression of studied gene (R) was calculated following the formula: R = Densitometrical Units of each studied gene/Densitometrical Units of β-actin (Table 2).

Statistical analysis
Data was coded and entered using the statistical package SPSS version 17 (SPSS, Inc., Chicago, Illinois, USA). Data were summarized using mean ± standard deviation for quantitative variables and percentage for qualitative variables. Comparisons between groups were made using χ^2 tests for qualitative variables and analysis of variance for normally distributed quantitative variables. Correlation was carried out to test the linear relationship between quantitative variables. A P value of less than 0.05 was considered statistically significant.

Results
Patient and control data
The study included 20 patients; 12 male (60%) and eight female (40%). Their age ranged from 10–46 years with a mean of 26.7 ± 10.9 years. Fifteen controls were included in the study, seven male (46%) and eight female (53%). Their age ranged from 15 to 42 years with a mean of 27.7 ± 7.6 years. Both patients and controls were age-matched ($P = 0.737$) and sex-matched ($P = 0.157$) (Table 3).

Results of the quantitative polymerase chain reaction measurement of Cx43 mRNA expression
The mean Cx43 mRNA expression in the patients was 1.23 ± 0.1 μg/gm tissue and 0.27 ± 0.05 in the control group. This denotes a significant statistical difference between the groups ($P = 0.000$) revealing higher expression in patients with atopic dermatitis (Fig. 3).

The highest level of Cx43 expression was found in patients with severe atopic dermatitis; however, the differences detected between patients with mild, moderate, and severe atopic dermatitis were not statistically significant ($P = 0.127$) (Fig. 4).

No statistically significant correlation was present between each of sex, age, duration of the disease, and CX43 expression.

Discussion
This study revealed a significant increase in Cx43 mRNA expression in atopic skin when compared with normal skin ($P = 0.000$). To the best of our knowledge no previous studies have detected any relationship between Cx43 and atopic dermatitis.

Cxs are the structural GJ proteins that form channels for the exchange of small molecules between connected

Table 3. Clinical data of patients with atopic dermatitis

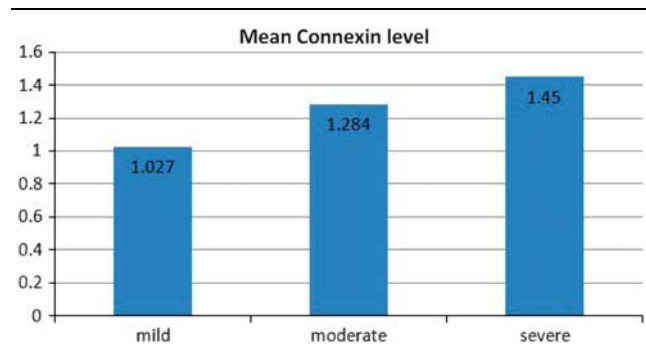| Number	Age (years)	Sex	Personal history[a]	Family history	Grade of severity (score)[b]
1	22	Female	–	Positive	Mild 4
2	41	Male	–	Negative	Moderate 6
3	18	Female	–	Negative	Mild 3
4	34	Male	Asthma	Negative	Severe 9
5	45	Male	–	Positive	Severe 8
6	21	Male	–	Negative	Mild 4
7	19	Female	Asthma	Positive	Severe 9
8	28	Male	–	Negative	Mild 4
9	13	Female	Asthma	Positive	Severe 8
10	10	Male	–	Negative	Mild 4
11	25	Male	–	Negative	Mild 4
12	33	Male	Asthma	Negative	Severe 9
13	46	Female	Asthma	Negative	Severe 9
14	19	Female	–	Positive	Mild 3
15	45	Male	–	Negative	Moderate 5
16	15	Female	–	Positive	Severe 8
17	23	Female	–	Negative	Mild 3
18	30	Male	Asthma	Negative	Moderate 4
19	28	Male	–	Negative	Moderate 6
20	20	Male	–	Negative	Moderate 5

[a]Personal history indicate the presence of history of other atopic manifestations such as atopic asthma.
[b]The grade of clinical severity was evaluated according to Rajka and Langeland [16]: mild (score: 3–4); moderate (score: 4.5–7.5); and severe (score: 8–9).

Figure 3.
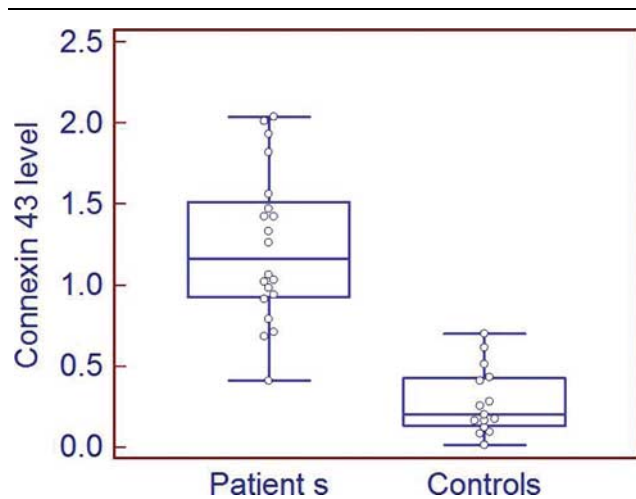
Expression of connexin 43 in patients and controls.

Figure 4.

Mean connexin 43 level in atopic patients of different degrees of severity.

cells. The cells efficiently use these GJs for electrical synapse and sharing of intracellular metabolites that coordinate various metabolic events [17]. Cx43 is located both in the interfollicular epidermis and in adnexal structures [18]. It is expressed by most of the immunocompetent cells including macrophages, neutrophils, mast cells, and lymphocytes [18]. It is also expressed along with Cx40 in polymorphonuclear cells under inflammatory conditions in humans [19]. The increased expression of Cx43 has been shown to increase the levels of various inflammatory and immunomodulatory molecules such as interleukin-1 and interleukin-6, transforming growth factor-β in cocultured endothelial cells, and smooth muscle cells [20]. Cx43 is known to be regulated by various factors such as glucose [21] and

nitric oxide [22]; and growth factors such as fibroblast growth factors [23], transforming growth factor-β [24], Epidermal growth factor [25], platelet-derived growth factor [26], nerve growth factor [27], ATP [28], and few cytokines such as tumor necrosis factor-α and interferon-γ [29] though the precise molecular mechanisms by which they regulate Cx43 are not fully understood.

TJs have been studied recently in atopic dermatitis. One study showed that claudin-1 expression (one of the proteins forming the TJ) is markedly reduced in atopic dermatitis skin and this suggests that the barrier defect in this disease may be at the level of TJ [30]. Some studies have shown that several growth factors that regulate GJ also regulate TJ permeability, whereas others have shown that Cxs and TJ tetraspan membrane core proteins, claudins and occludins, coimmunoprecipitate [31,32], and that GJs may even regulate the expression and function of TJ proteins [33,34]. In addition, some studies have emphasized an important role played by the TJ in

regulating the GJ size and stability. They found that blocking ZO-1 binding to Cx43 increases the size of GJs in HeLa cells [14], a position supported by ZO-1/Cx43 colocalization studies in cardiomyocytes [15].

Altogether, and from our results that showed higher expression of Cx43 in atopic patients, we hypothesize that the disease might start by downregulation of the TJ (which can be triggered either by extrinsic or intrinsic factors) leading to decreased binding to Cx43 and hence its upregulation. The increased level of Cx43 then excites an inflammatory response, as Cx43 has been shown to increase the levels of various inflammatory and immuno-modulatory molecules [20]. This explains the higher level of the protein in severe cases detected in this study, although the difference was not statistically significant. This could be more evident if we could increase the number of studied groups of patients with atopic dermatitis with different degrees of severity in future studies.

Another explanation, is that increased expression of Cx43 increased expression in the disease could be a primary event, and as it is reported that it actually regulates the expression and function of TJ proteins its increased expression will lead to defective TJ, resulting in an epidermal barrier defect that will contribute to the evolution of atopic dermatitis.

In contrast, some other studies have reported that any cytosolic stress inhibits the dislocation and degradation of different Cxs [35]. Some evidence indicates that allergic and inflammatory skin diseases such as atopic dermatitis, urticaria, and psoriasis are mediated by oxidative stress. Mast cells generate mainly intracellular reactive oxygen species following the aggregation of FceRI; these reactive oxygen species may act as secondary messengers in the induction of several biological responses [36]. This raised another possibility that the disease through inducing this form of oxidative stress on tissues, might lead to decreased degradation of Cx43 hence elevating its level and aggravating the inflammation.

In conclusion, we found that increased expression of Cx43 in atopic dermatitis, regardless of whether it has a primary or secondary role in the pathogenesis of the disease, certainly plays an important role in enhancing the inflammatory process. It might share other factors that cause the defect in the epidermal barrier function. These findings raise new future directions for the management of patients with atopic dermatitis.

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