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Serum Levels of Inhibitors of Apoptotic Proteins (IAPs) Change with IVIg Therapy in Pemphigus

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TO THE EDITOR

Pemphigus is a rare autoimmune blistering disease characterized by high levels of antibodies against the epidermal attachment components, desmoglein1 (Dsg-1) and desmoglein3 (Dsg-3; Amagai, 1999). Intravenous Ig (IVIg) reduces the levels of these antibodies, as well as clinical manifestations of pemphigus, but its mechanism of action is unknown (Czernik *et al.*, 2008). There is emerging evidence that pemphigus antibodies can cause keratinocyte apoptosis and contribute to acantholysis (Arredondo *et al.*, 2005; Li *et al.*, 2009; Schmidt and Waschke, 2009). Inhibitors of apoptotic proteins (IAPs) can target the caspases, and increases in their levels lead to resistance to apoptosis (Schimmer, 2004). Antiapoptotic effect of IVIg on keratinocytes is well known in disorders such as toxic epidermal necrolysis (Viard *et al.*, 1998). Our goal in this study was to determine whether IAP levels change in sera during IVIg therapy as a possible mechanism of suppression of acantholysis.

We measured three IAPs (survivin, livin, and X-linked IAP (XIAP)) in sera of seven patients with active pemphigus (five pemphigus vulgaris and two pemphigus foliaceus) before and after treatment with 10% IVIg (Gamunex, Talecris Biotherapeutics, Durham, NC). Average age of the patients was 59 (45–74) years. Each course of IVIg consisted of four cycles, administered every 2 weeks. Each cycle consisted of 400 mg kg^{-1} per day infused slowly for 5 days. In four patients, IVIg was administered together with 50–200 mg per day of oral cyclophosphamide. All patients were under treatment with prednisone (20–80 mg per day) before IVIg therapy. Sera were collected before treatment, 1 and 2 weeks after the first and 1 and 4 weeks after the fourth IVIg cycle. All patients gave written informed consent according to the Declaration of Helsinki Principles, and the study was approved by the New York University Institutional Review Board. Serum levels of intercellular IgG antibodies were measured by indirect immunofluorescence using esophagus of rhesus monkey as the substrate. Skin cryosections were incubated with serial serum dilutions of 1:10 to 1:1,280. Goat anti-human IgG-FITC was used as the secondary antibody and slides were analyzed with an epiluminescence microscope. Anti-Dsg-1 and anti-Dsg-3 antibodies were measured by ELISA, using a kit from MBL International, Woburn, MA, and monoclonal mouse anti-human IgG4 antibodies (GeneTex, Irvine, CA) with the technique described previously (Green and Bystryn, 2008). Disease severity was measured using a score described earlier (Lolis *et al.*, 2011). For quantitative measurement of IAPs, ELISA kits from R&D Systems, Minneapolis, MN were used according to the manufacturer's instructions.

Serum survivin, livin, and XIAP were measured in 31 healthy individuals, and the average levels were 40.1 pg ml^{-1} (SD = ± 8.4), 26.2 pg ml^{-1} (± 14.4), and 0.9 μg ml^{-1} (± 0.29), respectively. Pretreatment levels of XIAP and livin in pemphigus patients (Table 1) were not significantly different from those of healthy individuals. Survivin

Abbreviations: Dsg-1, desmoglein1; IAP, inhibitors of apoptotic proteins; IVIg, intravenous Ig; XIAP, X-linked inhibitor of apoptotic proteins

Table 1. Changes in average disease severity score, pemphigus antibody titers, and IAPs in seven patients with pemphigus during treatment with IVIg

	Pre-Tx	1-W post 1st IVIg cycle	2-W post 1st IVIg cycle	1-W post 4th IVIg cycle	1-M post 4th IVIg cycle
Survivin (pg ml^{-1}) ± SD (Δ%)	30.9 ± 8.18	33.6 ± 6.4 (8)	30.4 ± 5.3 (−1.8)	29.6 ± 4.8 (−4)	29.3 ± 5.1 (−5)
Livin (pg ml^{-1}) ± SD (Δ%)	15.7 ± 12.08	139.4 ± 18.7 (786)	86.7 ± 16.4 (451)	240 ± 21.4 (1427)	65.3 ± 14.4 (315)
XIAP (μg ml^{-1}) ± SD (Δ%)	0.9 ± 0.46	2.2 ± 0.62 (155)	1.5 ± 0.37 (78)	3.1 ± 0.51 (268)	1.3 ± 0.5 (58)
DSS ± SD (Δ%)	11 ± 2.2	9.7 ± 3.1 (−11)	9 ± 3.7 (−18)	8 ± 3.7 (−27)	9 ± 3.1 (−18)
IIF IgG ± SD (Δ%)	248.6 ± 32.2	180 ± 21.4 (−27)	77.1 ± 19.3 (−69)	100 ± 22.8 (−59)	72.9 ± 23.9 (−70)
Anti-Dsg-1 or -Dsg-3 IgG4 (U ml^{-1}) ± SD (Δ%)	40.2 ± 14.2	34 ± 14.2 (−15)	28.9 ± 11.1 (−28)	11.8 ± 7.4 (−70)	22.9 ± 12.2 (−43)
Total IgG (g l^{-1}) ± SD (Δ%)	11.2 ± 4.2	31.1 ± 5.6 (177)	23.2 ± 4.4 (107)	54.4 ± 11.3 (385)	19.2 ± 4.4 (71)

Abbreviations: Dsg, desmoglein; DSS, disease severity score; IAP, inhibitors of apoptotic protein; IIF, indirect immunofluorescence; IVIg, intravenous Ig; Tx, treatment; XIAP, X-linked inhibitor of apoptotic protein.

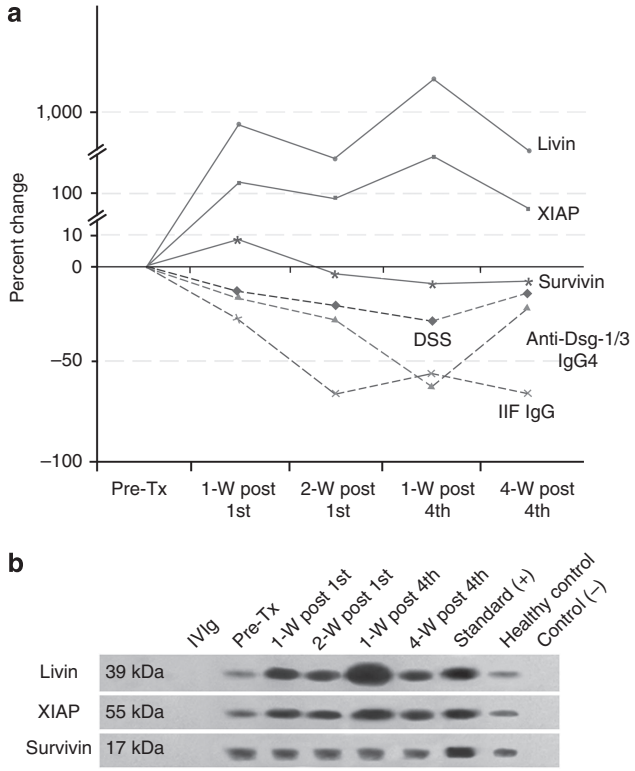

Figure 1. Serum levels of livin and XIAP increase during IVIg therapy for pemphigus. Changes in the average serum levels of inhibitors of apoptotic proteins (IAPs) measured by ELISA, disease severity score, and antibody titers in seven pemphigus patients during IVIg therapy (**a**), and western blot of serum IAPs in a pemphigus patient during IVIg therapy, and a healthy individual (**b**). For western blotting, 20 μg of protein was loaded on 10% SDS-PAGE gel and then transferred to nitrocellulose membrane. After blocking with 5% non-fat milk in Tris-buffered saline and 0.1% Tween 20, membranes were incubated with primary antibodies (goat polyclonal anti-human total livin, rabbit polyclonal anti-human survivin, and goat polyclonal anti-human XIAP, R&D Systems), followed by horseradish peroxidase-conjugated secondary antibodies. The immunoblots were developed using the enhanced chemoluminescence detection system. DSS, disease severity score; Dsg, desmoglein; IIF, indirect immunofluorescence; IVIg, intravenous Ig; Tx, treatment; XIAP, X-linked inhibitor of apoptotic protein.

was significantly higher in healthy individuals (P-value = 0.03). Analyses were performed using Student's t-test in Excel. One week after the first IVIg cycle, serum levels of livin and XIAP were markedly and significantly increased in all patients and, compared with the baseline, the average levels showed 786% and 155% increases, respectively (P-values = 0.002 and 0.039). Survivin levels remained balanced before and after treatment, and the difference was not statistically significant (Table 1). Two weeks after the first IVIg cycle, livin and XIAP levels were still higher than the baseline, with the greatest increase observed 1 week after the fourth IVIg cycle. Four weeks after the fourth IVIg cycle, livin and XIAP levels were still 315% and 58% higher than the baseline (Figure 1 and Table 1). To confirm ELISA findings, western blotting was also performed in three patients and the results were compatible with that of ELISA (Figure 1). In the IVIg preparation, levels of survivin, livin, and XIAP were 41.2, 22.3 pg ml^{-1}, and 0.59 μg ml^{-1}, respectively. These values were not significantly different from those of healthy individuals or patients before treatment. To evaluate the possible effect of IVIg contained in the test samples on IAP ELISAs, we measured serum total IgG before and after each IVIg cycle (Table 1). After the first cycle, IgG concentration increased by 177%. We concluded that a 786% increase in livin was less likely due to the impact of IVIg. One and four weeks after the last IVIg cycle, these differences were even more striking. Although changes in XIAP were close to those of total IgG, considering that livin and survivin changed independent of IgG, we conclude that the elevation of IAPs as analyzed by ELISA was not due to IVIg

contained in the test sera. Nevertheless, our findings do not allow to formally exclude a direct impact of IVIg concentration on the performance of our solid-phase assay. Furthermore, IVIg is a heterogeneous product with various effects and possible nonspecific bindings. Disease severity scores, levels of intercellular antibodies, and anti-Dsg-1 or -Dsg-3 IgG4 antibodies decreased during IVIg therapy, showing both laboratory and clinical improvement of the disease (Table 1 and Figure 1).

Evidence for effects of disease antibodies on apoptotic pathways have been observed in pemphigus. Increased expression of multiple caspases has been detected in lesions of pemphigus patients (Puviani *et al.*, 2003; Wang *et al.*, 2004). In skin cultures, activation of initiator caspases is seen after treatment with pemphigus IgG (Arredondo *et al.*, 2005; Schmidt *et al.*, 2009). Furthermore, administration of caspase inhibitors protects mice from developing blisters induced by pemphigus antibodies (Li *et al.*, 2009), which strongly suggests that caspase activation is critically involved in the formation of acantholysis. Livin and XIAP have similar Baculoviral IAP repeats inhibiting similar caspases and both have a really interesting new gene domain that has a role in protein ubiquitination, which can induce inflammation and immunity. Survivin is structurally different from other IAPs because it lacks the really interesting new gene domain. Survivin is known to function as a major factor in cytokinesis, contributing to the resistance of cancer cells to apoptosis (Richter and Duckett, 2000).

In summary, we found that serum levels of livin and XIAP increase markedly with IVIg therapy, which is accompanied by a decrease in the levels of pathogenic autoantibodies, as well as disease severity. These results indicate that increase in serum livin and XIAP may be associated with clinical benefits of IVIg. Although the sample size was small, the striking results we report may suggest performing functional studies on the role of IAPs in the mechanism of action of IVIg in preventing acantholysis. In addition, evaluation of IAPs in other IVIg-treated inflammatory disorders is useful in further understanding the mechanism of action of IVIg.

CONFLICT OF INTEREST

The authors state no conflict of interest.

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Selective Loss of Wild-Type p16^{INK4a} Expression in Human Nevi

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TO THE EDITOR

The p16^{INK4a} cyclin-dependent kinase inhibitor acts as a negative regulator of cyclin D-dependent kinases and is a critical gatekeeper at the G1–S checkpoint (Serrano *et al.*, 1996). Accordingly, p16^{INK4a} is frequently inactivated in human tumors, and deletions involving this locus occur frequently in melanomas. Inherited mutations in the *p16^{INK4a}* gene are also associated with melanoma susceptibility in 40% of multiple-case melanoma families (Goldstein *et al.*, 2006). It is generally acknowledged that the progressive and gradual