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ORIGINAL ARTICLE

Serum of patients with oral pemphigus vulgaris impairs keratinocyte wound repair *in vitro*: a time-lapse study on the efficacy of methylprednisolone and pyridostigmine bromide

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OBJECTIVES: Pemphigus vulgaris (PV) is an autoimmune blistering disease affecting primarily oral mucosa and skin. Among the drugs used for the therapy of pemphigus, both methylprednisolone (MP) and pyridostigmine bromide (PBr) can prevent acantholysis *in vitro*. However, their putative therapeutic properties in regenerating PV-like lesions and promoting the healing process still remain to be demonstrated. To address this issue, here we have developed a model for studying the process of epithelial cleft regeneration in PV by artificially wounding keratinocyte monolayers.

MATERIALS AND METHODS: The experimental model was established by scratching confluent monolayers to simulate the epithelial cleft; then, wound regeneration in the presence of submaximal concentrations of PV sera was studied by time-lapse microscopy, with or without the addition of MP and PBr in the culture medium.

RESULTS: Pemphigus vulgaris serum inhibited epithelial cleft repair of wounded monolayers. Indeed, in the presence of 10% (v/v) PV serum, keratinocytes reached only 2% confluence within 72 h *vs* an almost complete healing of controls. When administered together with PV sera, MP significantly ($P < 0.01$) enhanced wound fill by 30% after 72 h. PV-associated wound repair was significantly ($P < 0.05$) ameliorated by PBr by 24 h and keratinocytes reached 20% confluence after 72 h. Interestingly, neither MP nor PBr could accelerate wound healing when compared with untreated control monolayers.

CONCLUSIONS: In PV, MP and PBr exert their curative effects in part by enhancing the regeneration properties of keratinocytes. Indeed, our data suggest that both drugs can specifically counterbalance the detrimental effects of PV serum on keratinocyte wound healing. These findings provide an explanation for the efficacy of MP and PBr in the treatment of PV lesions in human skin and oral mucosa.

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Introduction

Pemphigus is a group of potentially fatal autoimmune blistering diseases affecting stratified squamous epithelia. The common lesion of all types of pemphigus is the disruption on cell–cell adhesion among keratinocytes with subsequent intraepithelial splitting, or acantholysis. Pemphigus vulgaris (PV) is the most common type of pemphigus, and in most (if not all) cases it begins within the oral cavity as multiple painful non-healing erosions. Then, in about one-half to two-thirds of the patients, the disease spreads to the skin (Bystryn and Rudolph, 2005). This means that in a significant number of cases, PV remains localized on mucous membranes. Mucosal dominant type PV shows predominant oral erosions with limited skin involvement, which are no more than five or six erosions or blisters (Amagai, 1999). Oral blisters are fragile and rupture readily, leaving erosions which heal with difficulty. Furthermore, gentle lateral pressure applied to an area adjacent to the affected site forms a blister, resulting in a positive Nikolsky's sign. Apparently, unaffected sites may also show blister formation after scraping. These features suggest that PV patients' skin/mucosa may have an impaired capacity of tissue repair after wounding.

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The array of keratinocyte self-antigens immunoprecipitated by circulating PV IgG includes desmoglein (Dsg) 1 and Dsg3, transmembrane glycoproteins which occur in desmosomes, and epithelial acetylcholine receptors (AChR; reviewed by Lanza *et al*, 2006). The mechanism by which acantholysis occurs and the precise event that finally causes epidermis to split, however, are still unclear. While PV autoantibodies bind to Dsg3 or AChR antigens in all areas of the skin and stratified squamous epithelium (mucosa), the disease occurs only in limited discrete areas. This observation would suggest that factors other than IgG must exist that are crucial to the formation of blisters and clinical manifestation of PV. Once the cleft occurs, however, such a new-formed epithelial wound cannot regenerate promptly, and the ulcer/erosion persists for long.

The morbidity and mortality from untreated pemphigus can be significant. Systemic corticosteroids have dramatically decreased the mortality rate for PV, but the complications of steroid therapy now drive the quest for improved non-steroidal regimes (Grando, 2006). Numerous studies on keratinocyte AChR have been allowing the development of novel therapies, with promising results, based on the anti-acantholytic effects of cholinomimetics drugs, such as pyridostigmine bromide (PBr) and carbachol (Grando and Dahl, 1993; Grando, 2004; Nguyen *et al*, 2004a), which are acetylcholinesterase inhibitors and act as indirect acetylcholine agonists. Treatment with PBr (360 mg day^{-1}) in patients with pemphigus allows for keeping the disease under control at a lower dose of systemic glucocorticosteroids (GS).

Wound healing is a dynamic balance between synthetic and degradative mechanisms. After cutaneous injury, a cascade of events is observed, which mediates tissue repair and eventually the re-establishment of the barrier function of the skin. Acute wounds normally heal in a very orderly and efficient manner characterized by four distinct, but overlapping phases: haemostasis, inflammation, proliferation and remodeling (Diegelmann and Evans, 2004). In PV lesions, disruption of epidermal cohesion occurs, basal cells shrink and separate from overlying cell layers, and finally blister develops (Bystryn and Grando, 2006). However, keratinocytes fail to successfully repair the injury in PV patients, and healing of the epithelial wound is delayed. The fact that plasmapheresis can rapidly recover skin/mucosal blisters (Turner *et al*, 2000) suggests that PV sera do contain factors that prevent blister healing. Similarly, corticosteroids are likely to exert their curative effects by acting directly on diseased tissue areas, as PV lesions heal early after commencement of steroids while titres of circulating PV IgG still remains high (Chryssomallis *et al*, 1995; Werth, 1996). However, it is interesting to note that GS can prevent but not reverse pemphigus IgG-induced acantholysis *in vitro* (Swanson and Dahl, 1983). This datum undermines the possibility of studying the therapeutic action of GS in cultured keratinocytes upon induction of cell-cell detachment by PV serum.

To overcome these limitations, here we have developed a model for studying the process of epithelial cleft regeneration in PV by artificially wounding keratinocyte monolayers. This method enabled us to compare the therapeutic effects of corticosteroids and cholinomimetic drugs during the healing process of PV-like lesions *in vitro*.

Materials and methods

Serum samples

Sera of PV patients and healthy donors were previously characterized (Cirillo *et al*, 2006; Lanza *et al*, 2008). Sera of two patients (PV1 and PV2) with only mucosal involvement were used for this study. These sera contained anti-Dsg3 IgG, but not anti-Dsg1 IgG, as revealed by Western blotting (Stellavato and Cirillo, 2007). ELISA scores for Dsg3 were 97 $\pm$ 7.2 (PV1) and 134.3 $\pm$ 11.9 (PV2).

Cell culture, wound production and treatments

A non-tumorigenic human keratinocyte cell line (HaCaT), which exhibits normal differentiation both *in vitro* and *in vivo* (Boukamp *et al*, 1988), was used in this study. Cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS), penicillin (50 U ml^{-1}), streptomycin (50 μg ml^{-1}) and fungizone (2.5 μg ml^{-1}) in an atmosphere humidified with 5% CO_2 (Cruz *et al*, 2007).

At the time of the experiment, cells were seeded and grown to confluence for 2 days in KAD (KGM and DMEM) medium (Lanza *et al*, 2007) in six-well dishes. Scratches were made with a sterile blue 1 ml pipettor tip perpendicular to the bottom of the dish. This allowed identification of three sites for each well at which migration was determined (see below). Afterward, cells were rinsed, and the wounded area was examined microscopically to ensure that cellular debris was removed. The wells then received fresh KAD medium with or without PV/control sera and/or drugs in various combinations, as reported in the following paragraph. The culture was photographed at each line/scratch intersection at zero time and again after 6, 12, 24, 48 and 72 h in at least two different experiments.

Methylprednisolone (0.25 mM) and PBr (0.25 mM) were diluted to their final concentrations in fresh KAD medium and administered alone or in combination with PV serum. Whole PV or control sera were diluted in KAD culture medium (10% v/v final concentration). When administered at 10% (v/v) final concentration, these PV sera are able to trigger PV-specific intracellular signaling although they cannot cause gross acantholysis *in vitro* (Lanza *et al*, 2008).

Time-lapse study

Time-lapse technique allows to observe continuously, through a microscope, the phenomenon that can last several hours or several days also. The system was designed to maintain all the required environmental conditions for cell cultures, namely: temperature (37°C), humidity (90–95%) and CO_2 (5%), thus allowing prolonged observations of cell events. The possibility

of visualizing several fields within the same sample ensured the reproducibility and the statistical significance of a single experiment. The temperature was controlled by circulating water from a thermostatic bath into the incubating chamber. A dedicated software was used for reading the temperature in a reference well and updating the set point temperature of the water bath, ensuring a temperature stability of ± 0.1°C. CO_2 was mixed with air in the control unit and was continuously fed into the incubating chamber to control medium pH. A humidifying and a preheating module prevented medium evaporation and avoided water condensation on glass and plastic surfaces. To perform Time-lapse experiments, OKO-VISION 2.7, an advanced imaging software designed to automatically control all the hardware components of microscopy workstation and to make image analysis (OKOLAB s.r.l., Ottaviano, NA, Italy), was used.

Statistical analysis

In our experiments, migration was determined at three sites for each well. Three independent experiments were recorded and subjected to statistical analysis by using Student's *t*-test (Cirillo and Boutros, 2008).

Results

Assessment of wound healing measurements and treatments

In pilot experiments, confluent monolayers were scratched with a sterile blue 1 ml pipettor tip and incubated with complete KAD medium plus 10% FBS. Three fields were chosen arbitrarily and the culture was photographed at each of the three line/scratch intersections at zero time and again after 6, 12, 24, 48 and 72 h. In these conditions, keratinocytes migrated progressively into a 0.5-mm scratch and 100% confluence was reached within 24 h (Figure 1).

To reduce the effect of cell proliferation on the healing process, we assessed the experimental conditions by culturing keratinocytes with serum-free KAD. In serum-free conditions, only 50% wounds were filled within 72 h (Figure 2). Next, we studied the healing process in the presence of 0.25 mM MP or PBr diluted in serum-free KAD. Cells exposed to PBr rapidly repaired the wound by 25% within 6 h. However, this effect was transient and the healing was $<40\%$ after 72 h. In the presence of 0.25 MP, keratinocytes gradually healed until reaching above 40% confluence after 72 h (Figure 2).

PV serum impairs normal wound healing of keratinocytes

We established a cell culture model for studying epithelial cleft regeneration in PV by wounding cultured keratinocytes and exposing them to PV serum. As seen in pictures shown in Figure 3, in some microscopy fields, keratinocytes reached about 100% confluence within 24 h when cultured in KAD medium plus 10% (v/v) control serum (Figure 3). The average of six measurements confirmed that sera of healthy individuals allowed an almost complete healing (Figure 3, *graphic*). In

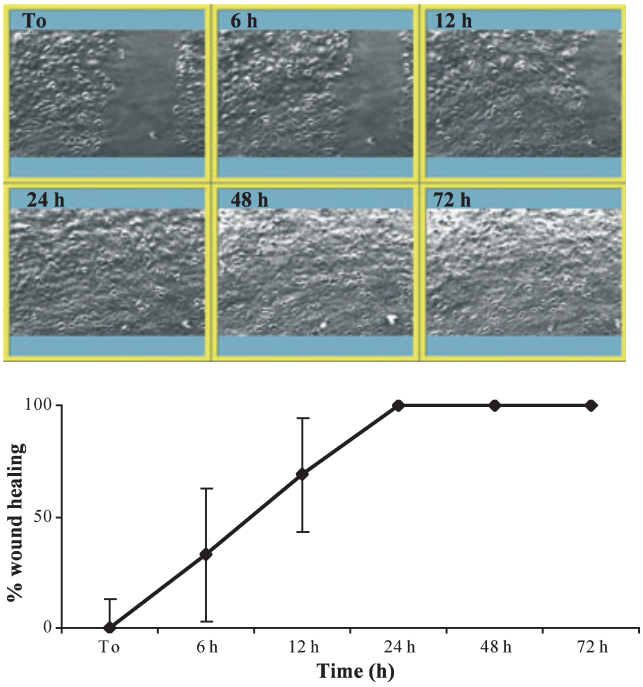

Figure 1 Wounded monolayers were photographed after scratching (time 0) and again after 6, 12, 24, 48 and 72 h. The graphic reports the mean ± s.e.m. of three different fields taken from the same experiment

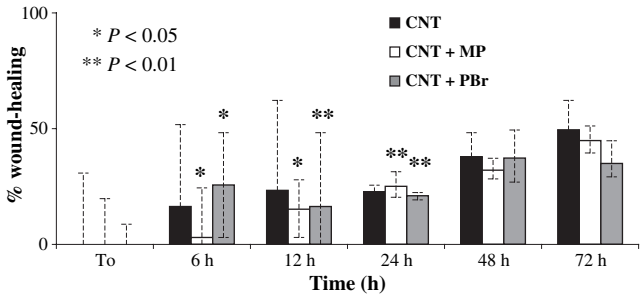

Figure 2 After wounding, cells were incubated with serum-free KAD medium alone or supplemented with either 0.25 mM methylprednisolone or 0.25 mM pyridostigmine bromide. Wound healing was calculated on the basis of six measurements from three independent experiments

marked contrast, PV1 serum exerted dramatic effects on wound repair, as keratinocytes were unable of migrating and filling the scratch (Figure 3); rounded keratinocytes appeared on cut's edges, and the percentage of healing was unappreciable (Figure 3). According to the mean values of three measurements, wound repair in PV1-treated cells peaked at 24 h with a 5% repair, then it decreased to 2% after 72 h (Figure 4). Measurements taken in three independent experiments by using PV1 and PV2 sera gave similar results (Figure 5).

Taken together, these data demonstrate that sera of patients with oral PV, but not of healthy individuals, contain factors that are able to prevent keratinocyte migration and repair of epithelial wounds.

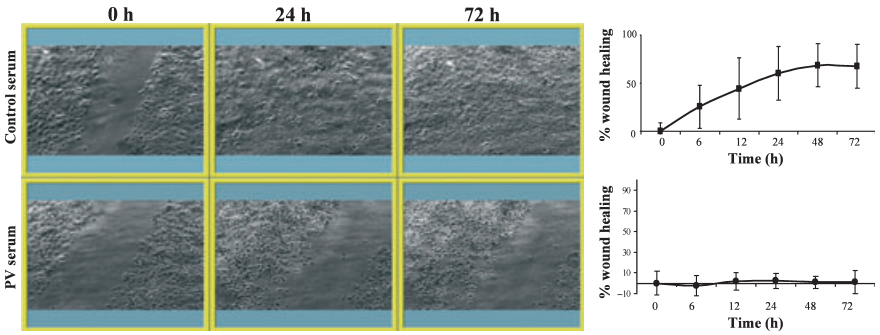

Figure 3 Monolayers were scratched as reported in materials and methods section a-nd then incubated with control or PV1 serum in KAD medium to 10% (v/v) final concentration. The results of six measurements in t-hree independent experiments with PV1 and PV2 sera are represented in the graphic

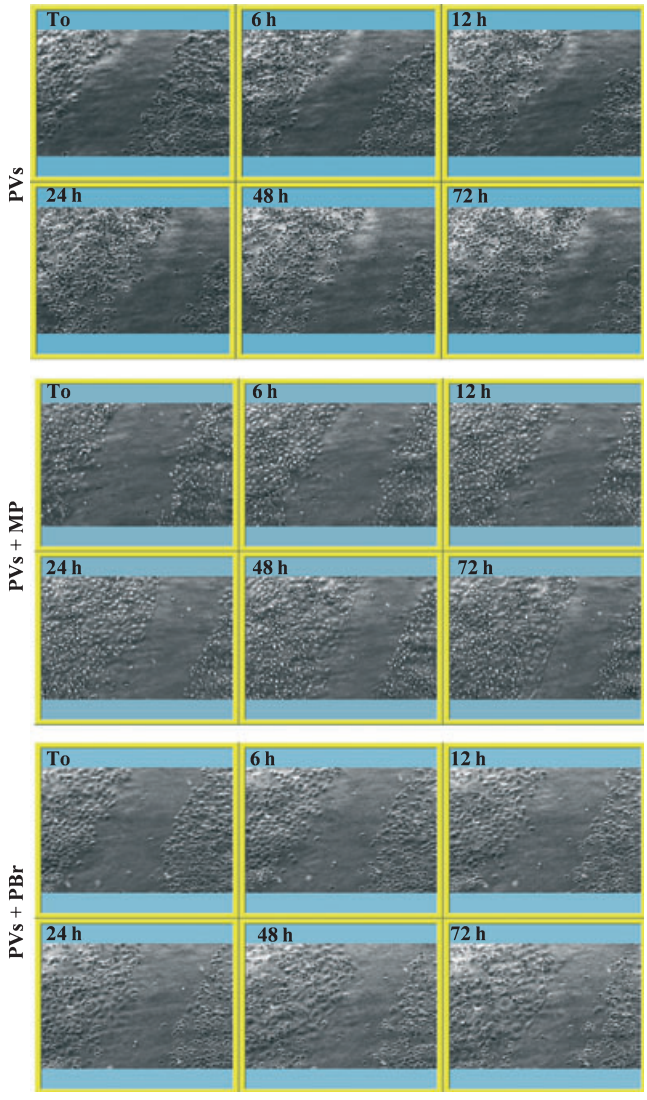

Figure 4 Time-lapse microscopy of healing monolayers in the presence of 10% (v/v) PV1 serum alone or supplemented with either 0.25 mM methylprednisolone or 0.25 mM pyridostigmine bromide. Photographs were taken at time 0 and after 6, 12, 24, 48 and 72 h

MP and PBr ameliorate wound regeneration affected by PV serum

We then tested whether MP and PBr could act by directly contrasting the detrimental effects of PV serum

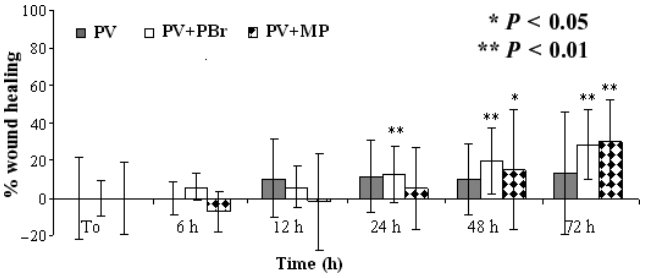

Figure 5 Histograms represent the mean ± s.e.m. of three independent experiments with PV1 and PV2 sera. Wounded monolayers were treated as in Figure 4

on keratinocytes, thus ameliorating their ability to repair epithelial wounds. When added to PV serum, 0.25 mM MP significantly ($P < 0.01$) enhanced healing of the scratch by 24 h (Figures 4 and 5). After 72 h, keratinocytes exposed to PV serum plus MP reached 30% confluence in comparison with only 2% confluence ($P < 0.01$) seen in monolayers treated with PV serum alone (Figure 5). In the presence of PV serum together with 0.25 mM PBr, keratinocytes underwent slow but progressive migration (Figure 4). The earliest significant changes were at 48 h ($P < 0.05$), and the scratch was filled by approximately 20% keratinocytes after 72 h (Figure 5). No statistical differences between monolayers treated with PV sera plus either MP or PBr were found. These findings indicate that MP and PBr are effective in promoting the regeneration of wounded keratinocytes in the presence of PV serum.

Discussion

It is well known that MP and PBr induce rapid healing of blisters in patients with PV, yet their ability to repair PV-associated epithelial cleft *in vitro* has never been demonstrated. In this study, we have shown that PV serum impairs keratinocyte wound regeneration, and that this detrimental effect is significantly improved in the presence of MP and PBr. Interestingly, both drugs could not accelerate wound repair in monolayers cultured under normal conditions. This suggests that MP and PBr specifically counterbalance the effects of PV serum on the healing process of wounded keratinocytes.

While corticosteroids and cholinomimetic drugs have been shown to ameliorate acantholysis and recover blisters *in vivo* (Nguyen *et al*, 2004a), their efficacy in

reversing the effects of PV serum *in vitro* is still controversial. This does not help understand the mechanisms of action of anti-acantholytic drugs and the pathways they interfere with. To study the therapeutic activity of MP and PBr in recovering PV-like lesions *in vitro*, here we have established an experimental model by scratching keratinocyte monolayers to simulate the detachment between different layers of the skin/mucosa (epithelial cleft). Then, the process of epithelial repair was followed by time-lapse microscopy in the presence of 10% PV serum alone (to simulate the healing of PV-like lesions), or in combination with the test compounds 0.25 mM MP or PBr (to study the curative effects of these drugs in the healing process). Results of this study have demonstrated for the first time that PV serum impairs the ability of keratinocytes of migrating and repairing an epithelial cleft. On the other hand, our data suggest that MP and PBr can exert their anti-acantholytic action by contrasting the arrest of keratinocyte regeneration induced by PV serum, thus improving the healing of PV-associated lesions.

Although corticosteroids are the mainstay in the therapy on PV, the exact mechanism through which they control the disease have not been fully elucidated. Modulation of immune system does not account for the early curative effects of GS, as blisters heal rapidly after the commencement of treatment while high antibody titres may last for 2–3 months. Local anti-inflammatory activity may reduce the afflux of IgG and cytokines, thus allowing epidermis to recover its integrity. The study by Sergei Grando has shown that MP and PV IgG exert reciprocal effects on keratinocytes (Nguyen *et al*, 2004b). Overall, PV IgG decreased transcription of 198 genes and increased transcription of 31 genes. MP decreased transcription of 14 genes and increased transcription of 818 genes. Reciprocal changes were also observed in terms of protein synthesis and phosphorylation of adhesion molecules (Nguyen *et al*, 2004b). Nguyen *et al* suggested that therapeutic effects of MP in PV include both genomic and the up-regulated synthesis and posttranslational modification of the keratinocyte adhesion molecules. Consistently, here we have demonstrated that MP may promote the healing of PV-like lesions by acting directly on keratinocytes, thus exerting opposite effects with respect to PV sera.

Elucidation of the cholinergic control of epidermal integrity has a potential for the development of treatment regimens using safer drugs to control blistering in a variety of oral and skin diseases, including pemphigus (Grando, 2006). Recent studies have been elucidating the molecular mechanisms allowing cholinergic agonists to inhibit PV IgG-induced acantholysis and phosphorylation of keratinocyte adhesion molecules. For instance, it has been demonstrated that the M_1 agonist pilocarpine ameliorated pemphigus acantholysis by inhibiting protein kinase C-dependent serine phosphorylation of β-catenins and tyrosine phosphorylation of p120-catenin via activation of protein phosphotase 2A and protein-tyrosine phosphatase, respectively (Chernyavsky *et al*, 2008). One of the hypothetical mechanisms that could explain the curative effect of cholinergic agonists in PV is the up-regulation of adhesion molecules protecting keratinocytes from cell–cell detachment. This mechanism would well explain the protective effect of cholinomimetic drugs in preventing blister formation, but not in healing the blisters in the presence of PV serum. Alternatively, or additionally, cholinergic agonists may work through direct competition with pemphigus IgG for binding to keratinocytes. Indeed, pemphigus IgG can cause the blockage of AChR with subsequent reduction of calcium influx. Our findings are consistent with the latter hypothesis, as it suggests that PBr acts, at least in part, by neutralizing the anti-regenerative activity of PV serum. The reciprocal effects of PBr and PV serum in the healing of epithelial wounds are confirmed by the finding that 0.25 mM PBr in KAD medium, i.e., in the absence of PV serum, could not improve or accelerate migration of keratinocytes in comparison with those cultured in KAD medium alone. Thus, PBr specifically counterbalances the effects of PV serum during epithelial cleft repair.

In conclusion, here we have provided evidence that corticosteroids and cholinergic agonists can control PV-like lesions *in vitro* via actions that harbor direct healing effects on keratinocytes.

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Author contributions

A Lanza designed the experiments and reviewed the manuscript. A Stellavato performed the experiments and analyzed the data. I Heulfe reviewed the manuscript. C Landi analyzed the data. F Gombos contributed to reagents and helped planning the additional experiments. N Cirillo designed the experiments, analyzed the data and wrote the manuscript.

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