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INVESTIGATIVE REPORT

Low-dose Oral Methotrexate Induces Apoptosis of Tissue Eosinophils in Bullous Pemphigoid

Kristina DAHLMAN-GHOZLAN[1,2], Jean-Paul ORTONNE[2], Philippe BAHADORAN[2], Anne SPADAFORA[2] and Eija STEPHANSSON[1]
Department of Dermatology [1]Karolinska University Hospital, Solna, Stockholm, Sweden and [2]Hospital Archet II, INSERM U385, Nice, France

We have shown previously that eosinophils are abundant in blood and tissue in bullous pemphigoid and that they rapidly disappear, without sign of necrosis, during methotrexate therapy. The aim of the present study was to investigate the role of apoptosis in this phenomenon. The terminal deoxynucleotidyl transferase-mediated dUTP-biotin nick-end labelling method for detecting apoptosis was used, combined with immunohistochemical detection of eosinophils before and during oral methotrexate. The numbers of apoptotic eosinophils were compared using light microscopy. Eosinophil apoptosis was significantly higher in skin after one week of methotrexate therapy and the number of eosinophils was reduced. The results indicate that methotrexate therapy induces apoptosis of the tissue eosinophils in bullous pemphigoid. ***Key words: apoptosis; skin eosinophils; bullous pemphigoid; methotrexate.***

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Kristina Dahlman-Ghozlan, Department of Dermatology, Karolinska University Hospital, Solna, SE-171 76 Stockholm, Sweden. E-mail: kghozlan@bredband.net

Bullous pemphigoid (BP) is an autoimmune blistering skin disease mainly affecting elderly people. Different auto-antibodies to the hemidesmosomal unit have been detected in serum. In histological skin sections a subepidermal inflammatory infiltrate rich in eosinophils is seen early in the disease (1). We have shown previously that the tissue eosinophils disappear rapidly during oral low-dose methotrexate (MTX) therapy of BP and that this event is reflected by diminished expression of the adhesion molecules VCAM-1 and E-selectin in skin and serum (2–4). The mechanism behind the prompt diminution of tissue eosinophils after immunosuppressive drug therapy in BP is not known. Apoptosis, physiological or programmed cell death, is a process for eliminating aged or damaged cells without causing inflammation, in contrast to necrosis (5). It is characterized by DNA fragmentation associated with morphological changes such as nuclear shrinkage and chromatin condensation in a single cell. The genetic regulation of apoptosis involves a complex gene system of suppressors as the Bcl-2 family, effectors such as caspases and intermediate genes such as Fas/ Fas ligand, p53, and Myc (6).

Inhibition of apoptosis plays an important role in autoimmune diseases (7). Immunosuppressive drugs such as MTX are efficient in many of these diseases.

The terminal transferase-mediated biotinylated 16-deoxy-uridine-triphosphate (dUTP) nick- end labelling (TUNEL) method is an *in situ* DNA fragmentation assay that can be used for detecting apoptosis in paraffin-embedded tissue sections (8).

The monoclonal antibody EG2 (Pharmacia & Upjohn, Uppsala, Sweden) has been evaluated largely in clinical and *in vitro* studies. EG2 identifies active and resting eosinophils by binding to eosinophil cationic protein (ECP) and eosinophil-derived neurotoxin (EDN) (9). In the present study we combined the TUNEL assay method with immunohistochemical staining of the eosinophils with the monoclonal antibody EG2, in order to investigate the role of apoptosis in eliminating the eosinophils from the skin during oral, low-dose, immunosuppressive MTX therapy of BP.

MATERIALS AND METHODS

Skin biopsies from 10 patients with generalized BP were investigated, prior to and after one week (mean 5.3 days) of oral low-dose MTX (2.5–5 mg/week). Double-staining for simultaneous detection of DNA fragmentation (apoptosis) of the eosinophils in the tissue was performed using a combination of the TUNEL method and immunohistochemical identification with a modification of the triple technique described by Bonkhoff et al. in 1999 (8).

Terminal transferase-mediated biotinylated 16-deoxy-uridine-triphosphate (dUTP) nick- end labelling (TUNEL) assay

Formalin-fixed paraffin-embedded tissue sections were dewaxed in xylene, rehydrated through graded ethanol and pretreated with proteinase-K (20 μg/ml) in phosphate-buffered saline (PBS) for 15 min at 37°C in a humidified chamber. After washing twice in distilled water and rinsing in Tris-buffered saline (TBS) (pH 7.6), the sections were incubated for 60 min at 37ºC with a reaction mixture containing 0.3 U/μl terminal deoxynucleotidyl transferase (TdT; Promega, Charbonniere, France), terminal transferase buffer (Promega): 100 mM cacodylate buffer (pH6.8), 1 mM cobalt chloride, 0.5 mM DTT (DL-dithiothreitol) with added biotinylated 16-dUTP (Roche Diagnostic, Meylan, France). The reaction was terminated by rinsing twice in TBS. Sections were then covered with 2% bovine serum albumin (BSA) in TBS for 15 min and then incubated

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with avidin-biotin-conjugated alkaline phosphatase (ABC-AP; DAKO, Trappes, France) at 1:100 for 30 min. Staining was achieved with 5-bromo-chloro-indoxyl phosphate/nitro blue tetrazolium (BCIP/NBT; Vector/AbCys SA, Paris, France) with 1 mM levamisol added to inhibit the endogenous alkaline phosphatase activity. The result gave a blue/purple colour to the nucleus in apoptotic cells.

A positive control was achieved by staining sections of rat mammary gland – ApopTag control slides (Appligene/Oncor, Illkirch, France). In the negative control, TdT was omitted from the reaction buffer.

Immunohistochemical staining of eosinophils with monoclonal mouse antibody EG2

The slides were blocked in normal rabbit serum (1:5) for 30 min and the EG2 monoclonal mouse antibody 100 µg/ml (Pharmacia & Upjohn) was applied at 1:200 for 60 min. Then a monoclonal non-biotinylated rabbit anti-mouse antibody (DAKO) was applied at 1:50 for 30 min. The alkaline-phosphatase anti-alkaline phosphatase (APAAP) complex (DAKO) was added at 1:50 for 30 min. Staining with the New-Fuchsin substrata (DAKO) with 0.2 mM levamisol resulted in a red staining of the cytoplasm of the eosinophils. The slides were faintly counterstained by methylen green (DAKO).

Negative control was achieved by replacing the primary antibody with a species such as IgG1 at the same concentration.

Cell counting in light microscopy

Two investigators (KDG and PB or KDG and ES) evaluated all the slides independently and blindly. Five light power fields at magnification ×40 were evaluated for each slide. All eosinophils identified as having red cytoplasmatic staining were counted.

The number of apoptotic eosinophils, identified as cells with positive double staining, was evaluated. Apoptotic eosinophils were defined as cells having a blue/purple nucleus with a typical morphology and a red cytoplasm.

Other apoptotic cells (cells with blue/purple typical nucleus) were noted.

Statistical analysis

The Wilcoxon's non-parametric test was used to evaluate the difference between cell counts of eosinophils before and after a single dose of oral methotrexate. p-values < 0.05 were considered significant.

RESULTS

Immunosuppressive monotherapy with oral low-dose MTX in 10 patients with generalized BP resulted in a reduced number of eosinophils ($p = 0.07$) in skin (Table I) after one week (mean 5.3 days, range 2–10 days). The mean number of eosinophils before therapy was 51.1 cells/field at × 40 light power fields (range 17.6–143). After one dose of MTX it was 33.8 cells/field (range 1.2–95.3) Elevated numbers of apoptotic eosinophils (mean 4.7 %, range 0–10.6% of total eosinophils) ($p < 0.05$) were seen in 6 of the patients after a mean of 4.2 days, (range 2–5 days) after a single dose of oral MTX (Fig. 1, patient 3). Skin samples showing no increased number of apoptotic eosinophils were collected later on (mean 7, range 4–10 days). Very few apoptotic eosinophils were identified (mean 0.08%, range 0–0.3% of total eosinophils) in skin samples from the patients with BP before MTX therapy. Apart from some apoptotic keratinocytes in the basal epidermal cell layer, few other apoptotic cells were detected.

DISCUSSION

The production of eosinophils in bone marrow is regulated by cytokines such as granulocyte macrophage colony-stimulating factor and interleukin-3 and terminal differentiation is promoted by IL-5. These cytokines can be produced by Th-type-2 lymphocytes, mast cells and by the eosinophil itself. In BP serum and blister fluid (10), and in skin samples (11), IL-5 is the predominant cytokine. The transmigration of eosinophils from the vessels to extracellular tissue is regulated by adhesion molecules, in allergic inflammation, especially by VLA-4/VCAM-4 present on eosinophils but not on neutrophils (12). We have previously shown high expression of VCAM-4 in active BP lesions and inhibition of this expression by MTX therapy. (3). IL-5 prolongs eosinophilic survival, and BP and allergic

Table I. *Eosinophils in the skin before and after one low dose of oral methotrexate*

Bullous pemphigoid patients	Eosinophils before MTX*	Eosinophils after MTX*	Days after first MTX dose	Apoptotic eosinophils before MTX* (% of total eosinophils)	Apoptotic eosinophils after MTX* (% of total eosinophils)
1	50.9	27.8	5	0	1.3 (4.7)
2	57.4	6.1	10	0.1 (0.2)	0
3	26	29.6	5	0	1.4 (4.7)
4	43.7	12	5	0.1 (0.2)	0.3 (2.5)
5	33.8	10	4	0	0
6	63.5	95.3	5	0	6.6 (6.9)
7	143	124.8	2	0.1 (0.07)	3.1 (2.5)
8	35.1	9	5	0.1 (0.3)	0
9	40.2	22.6	3	0	2.4 (10.6)
10	17.6	1.2	9	0	0
Mean	51.1	33.8	5.3	0.08%	1.51 (4.7)

*Number of eosinophils per light-power field at × 40.
MTX: methotrexate.

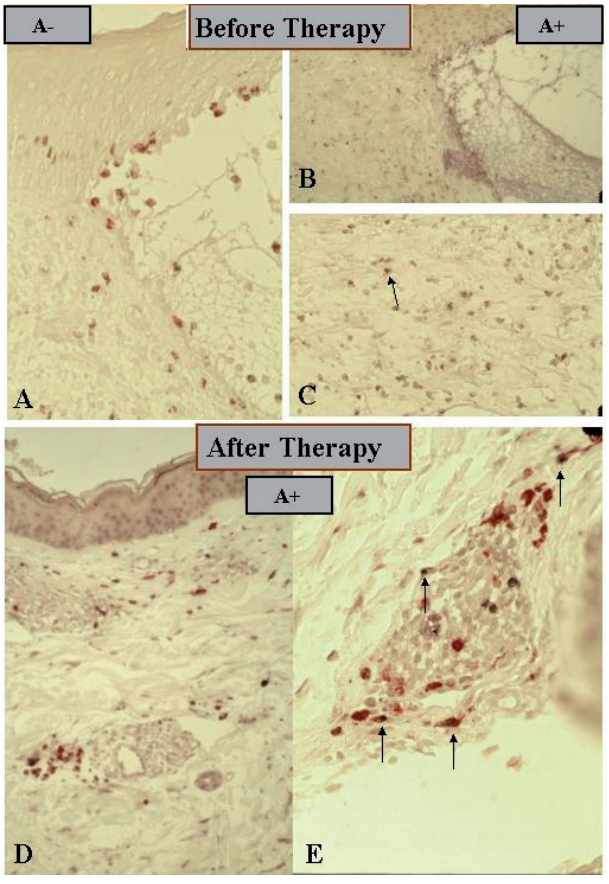

Fig. 1. Before methotrexate (MTX) treatment in patient 3, the negative control (A) shows eosinophils around subepidermal bullae, but in the double staining (B–C) there are no apoptotic eosinophils (*arrow*: ordinary eosinophil). In staining from the fifth day after one dose of oral MTX (D–E) 4.7% of the eosinophils show positive staining for apoptosis (*arrows* on double-stained cells around mid-dermal vessel). A–: the negative control for apoptosis with staining for eosinophils. Note that the enzyme terminal deoxynucleotidyl transferase –(TdT) is omitted from the TUNEL assay. A+: the double staining for apoptotic eosinophils.

diseases such as asthma and atopic dermatitis are often associated with high expression of IL-5 and accumulation of eosinophils in tissue. In allergic disease the corticosteroids disrupt the prolonged survival of eosinophils by inducing apoptosis and by suppressing the release of cytokines such as IL-5 (12–15). We detected no apoptotic eosinophils in samples from patients with BP before therapy; therefore it would be interesting to perform studies with markers of earlier events in the apoptotic process, such as caspases and inhibitors of apoptosis, to clarify whether the accumulation of eosinophils seen in BP is caused by inhibition of apoptosis. In earlier studies we have shown that MTX therapy down-regulates the expression of the adhesion molecules E-selectin and VCAM-1, thus decreasing the infiltration of eosinophils in BP. Our present results indicate that induction of apoptosis by MTX therapy might clear the eosinophils in the tissue without any further inflammation.

The tissue samples showing no increased eosinophil apoptosis were collected later on after the single MTX dose, indicating that MTX has a rapid effect and that eosinophil apoptosis might occur early. In our previous study (4), we showed that oral low-dose MTX has rapid distribution to the tissue (blister fluid) and reaches 30–40% of the peak serum concentration 1–3 h after intake. The mechanism responsible for eosinophil apoptosis during MTX therapy seen here in BP is not known. MTX has a potential to cause DNA damage, hence initiating apoptosis; but as no other significant signs of increased apoptosis were detected in the other cells in the tissue, DNA damage alone is probably not the mechanism responsible. It might act by diminishing the survival factor IL-5 responsible for accumulation of eosinophils, as glucocorticoids have been shown to do in allergic disease. To our knowledge this is the first study of the effect of MTX on eosinophils.

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