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INTERACTION BETWEEN CORTICOSTEROID BINDING GLOBULIN AND ACTIVATED LEUKOCYTES *IN VITRO*

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Summary: The interaction between human corticosteroid binding globulin and activated leukocytes is restricted to the granulocyte population, and is characterized by specific proteolytic cleavage of corticosteroid binding globulin which markedly reduces its steroid binding activity. A direct interaction between corticosteroid binding globulin and the activated cells appears to enhance this event, and does not involve cellular internalization of corticosteroid binding globulin or its proteolytic degradation products, which resemble those obtained after incubation of corticosteroid binding globulin with neutrophil elastase. These data suggest that corticosteroid binding globulin interacts with elastase on the surface of activated neutrophils, and may promote glucocorticoid delivery to these cells during inflammation. ©1990 Academic Press, Inc.

When administered in pharmacological amounts, synthetic glucocorticoids modulate the activities of several immuno-regulatory cell types to an extent that exceeds the anti-inflammatory effects normally associated with endogenous glucocorticoids (1). This may be attributed to the fact that increases in plasma glucocorticoid levels during inflammation are small when compared to the amounts of exogenous glucocorticoids required to obtain an anti-inflammatory response. There is, however, no doubt that natural glucocorticoids act locally as potent anti-inflammatory agents, and we have obtained evidence that human plasma CBG may promote cortisol delivery to sites of inflammation (2), as was originally suggested when it was discovered that CBG is a member of the serine protease inhibitor superfamily (3). This process appears to involve

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Abbreviations: CBG, corticosteroid binding globulin; PMA, phorbol-12-myristate-13-acetate; DMEM, Dulbecco's Modified Eagle's Medium; SDS-PAGE, sodium dodecylsulphate polyacrylamide gel electrophoresis; BSA, bovine serum albumin; A1-PI, alpha1-proteinase inhibitor.

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the cleavage of CBG by neutrophil elastase at a single location close to its carboxy-terminus (2,4), which results in a conformational change in CBG (4) and the release of bound steroid (2). We have therefore further examined the interaction between CBG and activated leukocytes, in order to determine how CBG may interact with neutrophils during inflammation.

MATERIALS AND METHODS

Human CBG was purified from late pregnancy serum by affinity chromatography, followed by ion exchange chromatography and gel filtration (5,6). Histopaque 1077 and 1119, and PMA were from Sigma Chemical Company. The culture medium was DMEM from Gibco Canada Inc., and contained 100 U penicillin/mL and 100 μg streptomycin/mL. Electrophoresis reagents and molecular weight markers were from Bio-Rad Laboratories (Canada) Ltd.

Separation and activation of leukocytes: Heparinized venous blood was applied to Histopaque gradients to separate mononuclear cells and granulocytes by centrifugation, according to instructions provided by Sigma. Separated cells were washed in 10 mL DMEM, and in some experiments aliquots of the separated cells were stimulated with 10^{-11} M PMA in 500 μl DMEM for 1 h at 37 C to promote leukocyte activation (7).

Interactions between CBG and activated neutrophils: To determine whether CBG interacts directly with neutrophils before elastase cleavage occurs, 2.5 x 10^5 granulocytes were isolated from blood samples taken for routine analyses from patients with sepsis. The cells were pre-incubated in DMEM (1 h at 37 C), and then separated from the "conditioned medium" by centrifugation (700 x g for 10 min) and resuspended in 500 μl DMEM. Human CBG (0.5 μg in 1 μL) was then added separately to the cells and the "conditioned medium", and both were re-incubated for 1 h at 37 C. Aliquots (10 μL) of the media in both cases were analyzed for CBG by western blotting (see below) and an established cortisol binding capacity assay (8).

We also examined the possibility that CBG accumulates within leukocytes during the incubations. To accomplish this, mononuclear cells (1 x 10^5), granulocytes (1 x 10^5), or a mixture of the same numbers of both cell types were first stimulated with PMA and then cultured with 0.5 μg CBG in 500 μl DMEM for 16 h. The activated cells were separated from culture media by centrifugation (700 x g for 10 min); cell pellets (harvested in approximately 10 μL without washing) and aliquots (10 μL) of the supernatants were then analyzed by western blotting.

Western blot analysis: Samples were resolved by SDS-PAGE (4% stacking and 12% separating gels), and proteins were electrotransferred (9) to an Immobilon^{TM} membrane (Millipore Corp.). After pre-incubation with 4% BSA-0.1% Tween-20 in TBS (50 mM Tris, pH 8.0, 150 mM NaCl), blots were incubated (16 h at 4 C) with a diluted (1:500 in TBS containing 0.5% BSA - 0.1% Tween 20) rabbit antiserum against human CBG (6). They were then washed several times in TBS, and immunoreactive proteins were detected with an alkaline phosphatase-labeled second antibody (Promega Biotec).

RESULTS AND DISCUSSION

In this study, we have confirmed that CBG only interacts with granulocytes within populations of naturally (Fig. 1) or artificially activated (Fig. 2) leukocytes, and the pattern of CBG degradation observed is similar to that obtained with purified neutrophil elastase (2). We have previously shown that cleavage of CBG can be protected when activated granulocytes are pre-incubated with a 10-fold molar excess of A1-PI to inhibit neutrophil elastase (2), and it is therefore reasonable to assume that this enzyme is responsible for the cleavage of CBG observed *in vitro*.

Neutrophil elastase is a cationic protein which tends to associate with cell membranes (10), and we have therefore addressed the question of whether a direct interaction between CBG and activated neutrophils is necessary before elastolytic cleavage occurs. The results of our experiments indicate that conditioned medium taken from naturally activated granulocytes, which would be expected to contain some free elastase, has little (Fig. 1, samples 3 and 4) or no (Fig. 1, samples 1, 2 and 5) capacity to degrade CBG. By contrast, when CBG was cultured under the same conditions in the presence of the activated cells, there was a substantial loss of CBG steroid binding activity in all samples (Fig. 1). The proteolytic fragmentation pattern varied remarkably between samples, and is indicative of different levels of protease production by granulocytes from different patients. For example, in samples 3 and 4, the degradation of CBG was remarkably efficient, and resulted in multiple, small molecular weight,

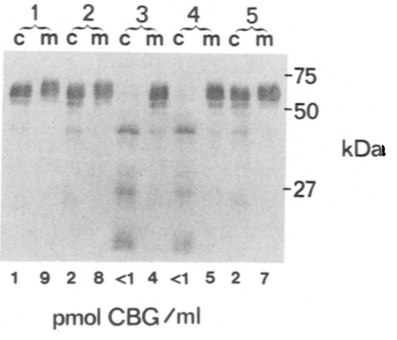

Fig. 1. Demonstration that CBG is cleaved by elastase upon direct interaction with spontaneously-activated granulocytes. Granulocytes from 5 separate patients (1-5) were pre-incubated for 1 h to obtain "conditioned media". The cells (c) and "conditioned media" (m) were then reincubated separately with human CBG (0.5 μg) for 1 h. Aliquots (10 μL) of the resulting culture media were resolved by 12% SDS-PAGE, transferred to Immobilon^{TM} membrane and developed with a CBG antiserum. Molecular size markers (kDa) are shown on the right. The steroid binding capacity of CBG in the culture media is indicated below.

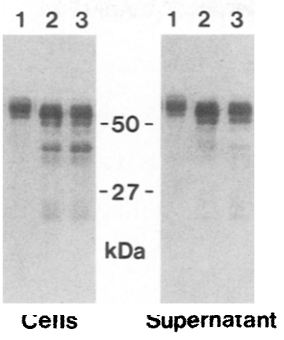

Fig. 2. Demonstration that CBG does not accumulate within monocytes or granulocytes upon proteolytic cleavage. Mononuclear cells (1), mononuclear cells and granulocytes (2), and granulocytes alone (3) were stimulated with PMA and cultured (16 h) with 0.5 μg human CBG. Cells were separated from culture media by centrifugation, and the entire cell pellet (left panel) or an aliquot (1/50) of the supernatant (right panel) was resolved by 12% SDS-PAGE; transferred to Immobilon^{TM} membrane, and developed with an antibody against human CBG. Molecular size markers (kDa) are shown between panels.

immuno-reactive fragments, similar to those obtained when CBG is incubated with an excess of purified neutrophil elastase (data not shown). On the other hand, the granulocytes in the other samples were far less aggressive in their ability to degrade CBG, but were certainly able to effectively reduce its steroid binding activity after only limited cleavage of the molecule, which essentially reduces its M_r by only approximately 5 kDa. These data indicate that proteolytic cleavage of CBG by naturally activated granulocytes, and the resulting loss of its steroid binding activity, occurs primarily as a consequence of a direct interaction with the cells, rather than by association with proteases released into the culture medium.

The fact that CBG in the culture medium may be entirely degraded by activated granulocytes within a 1 hour incubation period, suggests that this reaction probably occurs on the cell surface. To test this, we performed an experiment to assess whether CBG accumulates within activated granulocytes and/or monocytes during the brief incubation period required for CBG cleavage to occur in culture. When monocytes and granulocytes were isolated from a normal volunteer and activated with PMA, the granulocytes developed the ability to specifically cleave human CBG (Fig. 2) in a manner similar to that associated with granulocytes from patients with acute inflammation (Fig. 1). Monocytes, when activated in this way, however, are unable to degrade CBG and do not influence the ability of activated granulocytes to cleave CBG when co-cultured with them (Fig. 2). Moreover, when cells were harvested after incubation with CBG and

subjected to western blot analysis under denaturing conditions, the relative amount of CBG in the entire, unwashed cell pellet was approximately equal to that observed in only a fraction (1/50) of the culture supernatants (Fig. 2). These results confirm that internalization of CBG by either cell type is an unlikely event, and demonstrate that the presence of phagocytic monocytes do not influence CBG cleavage by elastase or interact with the proteolytic by-products.

It is becoming increasingly obvious that the relationship between CBG and other members of the serine protease inhibitor superfamily is not simply confined to similarities at the primary structural level (3). For example, we have recently demonstrated that the human CBG, A1-PI, and alpha1-antichymotrypsin genes are also closely related (11), and are all located on chromosome 14 in the q31-q32.1 region (12). When considered together, these data support the concept that these genes evolved relatively recently from a common ancestral precursor, and that this may have occurred to accommodate evolutionary refinements of a physiologically important function in the vertebrate species. It may therefore not be merely co-incidental that A1-PI and alpha1-antichymotrypsin play important roles in controlling neutrophil elastase and cathepsin-G, respectively, during inflammation (13), and our current data support the hypothesis that CBG may function in a similar physiological context with these two other serine protease inhibitors by promoting the delivery of anti-inflammatory steroids to activated neutrophils.

Acknowledgments

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