Patients with ulcerative colitis responding to steroid treatment up-regulate glucocorticoid receptor levels in colorectal mucosa

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\textbf{Keywords}
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\textbf{Abstract}

\textbf{Background and aims:} Glucocorticosteroid treatment (GCS) is effective for attacks of ulcerative colitis (UC). However, 25–30% of patients fail to respond and may be considered steroid resistant. Glucocorticoid receptors (GR) mediate the effects of GCS. Colorectal mucosa levels of GR and NF-κB were analysed before, during and after treatment with GCS-compounds.

\textbf{Methods:} Patients with moderate–severe attacks of ulcerative colitis were included. Patients undergoing colonoscopy with normal finding served as controls. GR and NF-κB levels in colorectal mucosa were analysed by Western Blotting and the DNA-binding activity of NF-κB by EMSA.

\textbf{Results:} Twenty-eight patients and seven controls were included. Ten patients were judged clinically steroid resistant. Responders had significantly higher levels of GR in colorectal mucosa after one week of treatment than non-responders \((P=0.039)\) and significantly higher levels of GR were found in responders in remission as compared to before treatment \((P=0.013)\). NF-κB levels did not differ between the groups at first visit. Increasing levels were found only in responders as remission was obtained \((P=0.031)\). EMSA detected 20% lower DNA-binding of NF-κB in responders in remission as compared to first visit \((P=0.021)\).

\textbf{Abbreviations:} GCS, glucocorticosteroid; UC, ulcerative colitis; GR, glucocorticoid receptor; NF-κB, nuclear factor kappa B; AP-1, activator protein 1; EMSA, electrophoretic mobility shift assay; SDS, sodium dodecyl sulphate; EDTA, ethylenediaminetetraacetic acid; HEPES, N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid; NR, non-responder; R, responder; GRE, glucocorticoid responsive element; PBMC, peripheral blood mononuclear cells; TPN, total parenteral nutrition.

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1. Introduction

Ulcerative colitis (UC) is a chronic inflammatory disease confined to the colorectal mucosa. In 1955 Truelove and Witts demonstrated that cortisone was efficacious in the treatment of UC. Glucocorticosteroids (GCS) are highly effective in the control of UC and other chronic inflammatory or immune diseases and these pharmaceutical compounds have remained first line treatment for active severe to moderately severe UC for more than 50 years. Several independent studies however, have demonstrated that 25–35% of patients with UC respond poorly or not at all to high doses of GCS. Factors governing response rate to GCS treatment include severity, duration and extent of disease. It seems unlikely though, that these factors alone could explain steroid resistance, since lack of response to GCS can also be seen among patients with mild distal UC. Steroid resistance is also found in other diseases involving inflammation such as asthma, rheumatoid arthritis and transplant rejection. The effects of GCS are mediated by the glucocorticoid receptor (GR). Previous studies have demonstrated that the number of receptors in a cell correlates well with the number of receptors in a cell. This study was undertaken to evaluate if the levels of GR and NF-κB correlated with the ability of active UC to respond to GCS treatment and also assess the longitudinal changes taking place during episodes of acute UC treated with GCS.

2. Materials and methods

2.1. Patient selection and treatment

Twenty-eight patients treated for moderate to severe acute attacks of UC at Karolinska University Hospital, Huddinge were included in the study. Seven patients undergoing colonoscopy for reasons other than inflammatory bowel disease (IBD) and with normal findings, served as controls. No patient was included more than once. All patients were seen in the out-patient IBD-clinic. A rigid sigmoidoscopy or colonoscopy for reasons other than inflammatory bowel disease were collected and stored at −80 °C until use.

2.2. Tissue

Two to four biopsies were taken from the rectal area and immediately snap-frozen in liquid nitrogen and stored at −80 °C.

2.3. Whole cell extract preparation

The biopsies were homogenised on ice in 100 μl of high salt EPG buffer (1 mM EDTA, 20 mM phosphate buffer pH 7.4, 10% glycerol, 0.4 M NaCl 5 mM dithiothreithol) containing protease inhibitors (2 ng/μl aprotinin, 1 ng/μl pepstatin, 2 ng/μl leupeptin, 100 ng/μl TPCK, 50 ng/μl TLCK) to extract both cytocolic and nuclear proteins and the supernatants were collected and stored at −80 °C.

2.4. Western Blotting

Bradford protein determination was performed for the cytosol prepared from the biopsies, and 20 μg of protein from each sample was separated on a 7% SDS gel. All samples from one patient were run on the same gel. Five, ten, fifteen and twenty microliters of diluted HeLa S3 extract prepared in 0.4 M NaCl containing EPG buffer (c.f. above) was included as a standard to be used in semi-quantitative analysis of the amount of GR. The HeLa cytosol was from the same batch, and was stored at −80 °C in small aliquots. Western Blotting to Hybrid Extra 0.45 micron nitrocellulose membrane (Amersham) was assessed with 0.1% (w/v) Ponceau S in 5% acetic acid (v/v) staining (Sigma, St. Louis, MO, USA) to confirm proper transfer and equal amounts of protein in all lanes.

Conclusion: GR levels increase in UC-patients responding to GCS-therapy but not in steroid resistant patients and may be the reason for the lack of steroid-efficacy. Increasing NF-κB levels were found in responders attaining remission, possibly reflecting a lower turnover. A decrease in DNA-binding of NF-κB was found in these patients, perhaps because of the increased GR levels counteracting NF-κB activity.
2.5. Immunostaining for GR and NF-κB

The nitrocellulose membranes were blocked in 10% non-fat dry milk-solution and incubated for 2 h at room temperature with primary antibodies directed against either GR (sc-1003, Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA) or p65 (sc-109, Santa Cruz Biotechnology Inc., Santa Cruz, CA), each diluted 1:5000 in PBS and 1% defatted dry milk. The membranes were washed 5 × 5 min in 0.05% Tween–PBS and then incubated for 1 h in room temperature with the secondary, horseradish peroxidase-coupled secondary antibody, polyclonal anti-rabbit IgG from donkey (Santa Cruz) diluted 1:5000 in PBS and 1% defatted dry milk. After 3 × 5 min washing in 0.05% Tween–PBS and 2 × 5 min in TBS, the luminol substrate SuperSignal® West Femto Maximum sensitive substrate, Pierce, was added to the membranes according to the description provided by the manufacturer.

2.6. Quantification

The photons generated in the enzymatic reaction were detected using the Fuji-LAS 1000 camera. Exposure time was set to about three times the exposure that gave clear bands on photo film and dark-frame subtraction was used. Analysis of the result was performed with the software Image Gauge V3.0. The brightness/contrast settings could be adjusted for optimal visual conditions to work with the image without altering the result. The signals from the dilution of HeLa cell cytosols were used to make a standard curve correlating amount of HeLa protein to signal. The signal from the samples could through this standard curve be used to determine how much HeLa cells protein that would yield the same signal as the 20 μg of protein sample. The GR content in the samples was expressed as percent of GR content in the same amount of HeLa cell protein.

2.7. Electromobility shift assay of NF-κB

Only biopsies from patients were analysed because of limited amounts of tissue available.

Whole cell extracts were prepared as described above in the section for cytosol preparation. Extract containing 4–8 μg total protein was added to a 20 μl binding reaction containing 20 mM HEPES pH 7.8, 5 mM MgCl₂, 100 mM KCl, 20% glycerol, 0.1% Triton X-100 and 0.1–0.3 ng 32P-labeled consensus oligonucleotide corresponding to the NF-κB binding site from the human ICAM-1 gene promoter (upper strand 5′-GGA TTC TGG AAA TTC CCT TT-3′) or in the case of AP-1 an oligonucleotide corresponding to an AP-1 consensus site (upper strand 5′-CGC TTG ATG ACT CAG CCG GAA 3′). Supershifts were conducted by including 2 μl of the p50 antibody sc-114, the p65 antibody (sc-109X), the c-jun antibody (H-79, sc-1694) or the c-Fos antibody (sc-52) (all commercially available from Santa Cruz Biotechnology Inc., Santa Cruz, CA) in the binding reaction. Specific and non-specific competition experiments were performed by including a 100-fold excess of non-labeled consensus oligonucleotide or the consensus oligonucleotide mutated at one base. The reactions were incubated for 20 min at room temperature and subsequently run on a PAGE (4% polyacrylamide, 0.25× TBE (1×; 90 mM Tris–borate, 2 mM EDTA), 0.01% NP-40). After the electrophoresis, the gels were dried and autoradiographed to film. Optical quantification of the electrophoretic mobility shift as demonstrated on the film was performed using the Fuji-LAS 1000 camera in light transmission mode after a semi-automated selection of the scanned area. The intensity of the major specific shifted fraction at visit one was set to arbitrary value 100%. The shifted fraction in the biopsies from the following visits, included on the same EMSA gel, were related to this value to see changes over time in NF-κB DNA-binding ability for each individual patient.

2.8. Statistical evaluation

Non-parametrical tests were used for the statistical analyses. For evaluation of longitudinal changes within a group, the paired sign rank test was used and for comparison of the different groups, the Mann–Whitney U-test was used. The statistical analysis of the data was performed with Statview software (Abacus Concepts, Inc., Berkeley, CA, USA). P-values below 5% were considered significant.

2.9. Ethical consideration

The study protocol was approved by the ethical committee of Karolinska University Hospital, Huddinge, and written informed consent was obtained from the participants.

3. Results

3.1. Patient characteristics and response to treatment

Twenty-eight patients and seven controls were included in the study. The patient characteristics are shown in Table 1.

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Responder</th>
<th>Non-responder</th>
<th>Controls</th>
</tr>
</thead>
<tbody>
<tr>
<td>N</td>
<td>18</td>
<td>10</td>
<td>7</td>
</tr>
<tr>
<td>Median age, year (range)</td>
<td>43 (22–67)</td>
<td>32 (20–59)</td>
<td>30 (22–67)</td>
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<tr>
<td>Sex (M/F)</td>
<td>12/6</td>
<td>8/2</td>
<td>4/3</td>
</tr>
<tr>
<td>Duration of disease, year</td>
<td>First n=8</td>
<td>n=3</td>
<td></td>
</tr>
<tr>
<td></td>
<td>0–2 n=3</td>
<td>n=1</td>
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<tr>
<td></td>
<td>3–5 n=1</td>
<td>n=2</td>
<td></td>
</tr>
<tr>
<td></td>
<td>&gt;5 n=6</td>
<td>n=4</td>
<td></td>
</tr>
<tr>
<td>Extent of disease</td>
<td>10</td>
<td>2</td>
<td></td>
</tr>
<tr>
<td>No. of patients with pan- or left-sided colitis</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Severity of attack</td>
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<td>n=4</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Moderate n=6</td>
<td>n=6</td>
<td></td>
</tr>
<tr>
<td></td>
<td>n=13</td>
<td></td>
<td></td>
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<tr>
<td>Initial treatment (n)</td>
<td>Systemic GCS</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>2</td>
<td></td>
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<tr>
<td>Enema:</td>
<td>Prednisolone</td>
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<tr>
<td></td>
<td>Hydrocortisone</td>
<td>2</td>
<td>3</td>
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<tr>
<td></td>
<td>Budesonide</td>
<td>2</td>
<td>1</td>
</tr>
<tr>
<td>Maintenance treatment (n)</td>
<td>SASP</td>
<td>3</td>
<td>2</td>
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<tr>
<td></td>
<td>Olsalazine</td>
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<td>2</td>
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<tr>
<td></td>
<td>Asacol</td>
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<td>0</td>
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<td></td>
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<td>12</td>
<td>6</td>
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Eighteen patients were defined as responders (R) and ten patients were defined as non-responders (NR). Six of the R and four of the NR had maintenance treatment with 5-ASA, but none of the patients had azathioprine or 6-MP.

One patient with a first attack of left-sided UC had a colectomy performed because of rapid deterioration despite treatment with TPN, i.v. betamethasone and prednisolone-enemas. Another patient in a similar situation, had a response that was slower than 10 days, but was reluctant to surgery, and colectomy was avoided because of firm improvement shortly thereafter. The remaining NR, primarily treated with topically applied GCS had a slow response to treatment and did not meet the criteria for response.

Figure 1  GR Western Blotting. Typical GR Western Blotting on rectal mucosal biopsies obtained from two individual patients. Lanes 1–4 are HeLa cytosol used as standard. Lanes 5–6 are samples from rectal mucosa in an individual before and after treatment until remission was obtained. Lanes 7–8 are samples from another individual before and after treatment. Lane 9 shows Western Blotting on biopsies from the same rectal area in a following attack of UC in this individual (but the same patient was only included once in the study-material).

Figure 2  GR levels in Western Blotting shown as box-plots. Boxes margins consist of 25th–75th percentiles. Median values are also marked within the boxes as a horizontal line if values exceed zero. The whiskers line out the 90th percentiles. The Western Blots were semi-quantified with HeLa S3 cytosol as standard. Optical densitometry was used to validate GR content as percent of GR content in the same amount of HeLa cell protein. In order to secure data about longitudinal changes in GR levels in individual patients, all samples from one patient were run on the same gel. A trend towards lower GR levels in patients as compared to controls was noted, but was not found to be statistically significant. GR levels increased significantly in R during the course of treatment from a median of 0 to 6.7 Arb. U. Higher levels in R than in NR were found at visit 2 (5.2 vs. 0 Arb U). Median GR levels in NR on the other hand remained at 0 Arb U on all visits. The subgroup “NR in remission” consists of the four patients among NR who eventually obtained remission.

Figure 3  NFκB levels in Western Blotting shown as box-plots. Median values are also marked within the boxes as a horizontal line if values exceed zero. As in Fig. 1, all samples from individual patients were run on the same gel, HeLa S3 cytosol was used as standard and optical densitometry for semi-quantitation. No differences in longitudinal development of NFκB levels in the studied groups were found at first two visits. In remission, a significant increase in NFκB levels, from 0 to 11.2 Arb U. was found in R. The subgroup “NR in remission” consists of the four patients among NR who eventually obtained remission.
3.2. Glucocorticoid receptor

Semi-quantitative Western Blotting (as described in the Materials and methods part) was used to determine GR levels relative to the standard from HeLa cells and a representative blot is shown in Fig. 1. We analysed GR levels in all patients at visit one (before commencing treatment), visit two (after one week) and after remission had been obtained, but with ongoing treatment. The results are shown in the box-plot in Fig. 2. No differences in GR levels were found between responders (R) and non-responders (NR) in inflamed rectal mucosa prior to treatment. Patients tended to have lower levels than controls, but this trend did not meet statistical significance at the 5% level (\(P=0.15\)).

After one week of treatment, R were found to have increased GR levels from 0 to 5.2 Arb U (\(P=0.039\)). In NR however no increase could be detected at visit 2 and median GR levels remained at 0 Arb U. GR levels in R were significantly higher at this point of time (\(P=0.039\)). R were found to have significantly higher levels of GR in rectal mucosa in remission as compared to inflamed mucosa at first visit (6.7 vs. 0 Arb U) (\(P=0.013\)) and obtained levels were not significantly different from healthy controls.

Four of the NR eventually obtained remission after step-up medication with oral prednisolone in three cases and lidocaine–gel combined with mesalazine enemas in one patient. No increase in GR levels could be verified in this subgroup of patients in biopsies obtained in remission as compared to before treatment.

3.3. Nuclear factor kappa B

The pro-inflammatory NF-\(\kappa\)B protein was assessed in two ways. First the amount of NF-\(\kappa\)B protein in whole cell extracts was estimated by semi-quantitative Western Blotting in a similar fashion as described for GR and secondly the amount of NF-\(\kappa\)B that had been activated and could bind to DNA was determined by EMSA.

NF-\(\kappa\)B levels as assessed by Western Blotting (Fig. 3) did not differ between R, NR and C at first visit. Only R had a significant increase at follow up as remission was obtained (0–11.2 Arb U) (\(P=0.0013\)).

In order to evaluate NF-\(\kappa\)B EMSA from colon biopsies we first performed a specificity control of the EMSA using material from one patient. As shown in Fig. 4 both probe specificity controls and supershift experiments using antibodies to both p65 and p50 ensured that we measured NF-\(\kappa\)B binding to the ICAM-1 promoter derived probe. In Fig. 5 we show a typical EMSA pattern from a patient first when inflamed and later...
eventually obtained remission. This is the first study undertaken to evaluate longitudinal changes in GR- and NF-κB levels in one and the same patient during acute attacks of UC treated with GCS. The main strength of a longitudinal study of this type is that possible demographical differences in the study-populations become less important. NR had a higher proportion of patients with severe disease, but only two of ten patients had inflammation beyond the splenic flexure compared to ten of eighteen of R.

Patients who responded well to treatment had higher levels of GR in rectal mucosa already after one week of treatment. They also had higher levels of receptors in remission than they had at first visit, before GCS treatment. NR, on the other hand, had low levels of GR in rectal mucosa at all visits. Four of the NR, eventually entered remission after having switched therapy. This subgroup did not increase GR levels significantly and receptor levels in all but one patient remained at 0 Arb U. Lower levels of GR seem to be related to active, untreated inflammation and poor clinical response.

The effects of GCS are mediated by the glucocorticoid receptor (GR) and affects the transcription rate of target genes. We know from previous studies that the number of GR in a cell correlate well with the amplitude of the cellular response to GCS.

GR and the pro-inflammatory transcription factor NF-κB have been found to interact with one another, and a mutual inhibitory effect has been demonstrated. Studies of GR in UC have given somewhat contradictory results. Most of the hereto presented studies have used ligand binding assays for the study of receptor levels. GR density in peripheral mononuclear cells in patients with active UC has been found to be up-regulated, or equal compared to controls. One study suggested that patients not responding to GCS treatment had lower GR levels in peripheral blood mononuclear cells. GR levels in colorectal mucosa in active UC, have been evaluated previously in two studies, demonstrating lower GR levels than in control-subjects with non-inflamed mucosa and lower levels in patients responding poorly to treatment.

The GR is known to be expressed in two isoforms, GRα and GRβ. The GRβ does not have transcriptional activity and is expressed in low concentrations (0.2–0.3% of the GRα). The role of this isoform has recently been debated. Using co-transfection systems, with supraphysiological levels of GRβ, this isoform of the receptor has been shown to down-regulate the GRα transactivating properties. Honda et al. also demonstrated GRβ to be present in PBMCs of steroid-refractory patients with ulcerative colitis significantly more often than in steroid-sensitive patients or controls. The precise meaning of this finding is still unclear since no transrepressive properties of GRβ have only been demonstrated with high concentrations of this isoform of the GR.

The lack of GCS-response in certain UC-patients may be due to a defective up-regulation of GR expression in effector-cells or decreased basal levels of GR or both. It is well known that GR are down-regulated in response to GCS. There are however also several reports on positive auto regulation. Ramdas et al. for example found basal level GR expression inadequate to mediate GCS-induced apoptosis in GCS-sensitive T-cells where positive auto regulation was shown to be a necessary component to achieve programmed cell-death. It is possible that the same mechanism of GCS-induced apoptosis of pro-inflammatory effector-cells is important for clinical response to treatment with GCS in UC. The longitudinal increase in GR levels in colonic mucosa in patients responding to GCS treatment could therefore reflect positive auto regulation of GR in activated T-cells subsequently undergoing apoptosis. Higher levels of GR may facilitate treatment efficacy creating a positive reinforcement of anti-inflammatory action. Patients with GCS-refractory disease did not increase their mucosal GR levels.

**Figure 6** NF-κB EMSA. Semi-quantitative analyses of intensity of shifted, radioactively labeled NF-κB oligonucleotides in an EMSA. Results are shown as box-plots. Median values are also marked within the boxes as a horizontal line if values exceed zero. NF-κB DNA-binding was defined as 100% at visit 1. Subsequent EMSA levels show differences (in %) related to this original value. Significant, longitudinal changes, as assessed by paired sign rank test, were noted only in R in remission, where a 20% reduction in NF-κB DNA-binding was found. The subgroup “NR in remission” consists of the four patients among NR who eventually obtained remission.

when the patient was in remission. In the gel-shift analyses of all patients we found a significant reduction by 20% of NF-κB binding only in responders obtaining remission (P = 0.021) (Fig. 6). No statistical significant reduction of NF-κB binding was found at visit 2 in R or NR.

4. Discussion

This is the first study undertaken to evaluate longitudinal changes in GR- and NF-κB levels in one and the same patient during acute attacks of UC treated with GCS. The main strength of a longitudinal study of this type is that possible demographical differences in the study-populations become less important. NR had a higher proportion of patients with severe disease, but only two of ten patients had inflammation beyond the splenic flexure compared to ten of eighteen of R.
The levels of NF-κB were not significantly different between controls and patients at first visit. Increased protein levels of NF-κB were found in responders obtaining remission, but not in the four non-responders who subsequently did respond to treatment. The DNA-binding activity of NF-κB was analysed by EMSA. The technique used did not allow us to compare different patients with each other but could validate longitudinal, individual changes in NF-κB activity. Responders were found to have a 20% median reduction of NF-κB DNA-binding ability as clinical and endoscopical remission was obtained when compared to first visit. It could be speculated that the subsequent increase in NF-κB levels in patients responding to treatment could be due to a diminished turnover of the transcription factor. It is also possible that the increased GR levels found in responders (but not non-responders) could decrease the DNA-binding activity of NF-κB noted in the gel-shift assay.

We conclude that patients with active ulcerative colitis have low levels of GR in rectal mucosa. After remission has been obtained, GR is up-regulated in rectal mucosa but these changes can be seen already after one week of treatment and could have implications for choice of treatment, at least in distal UC. NF-κB/p65 increases as remission is obtained in patients with an appropriate response to GCS treatment, but the ability of NF-κB to bind to an NF-κB response element decreases. Future studies to analyse levels of GR and NF-κB on a cellular level in immunological effector-cells, endothelial and epithelial cells are needed to further explore the findings in this study.

Acknowledgements

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