Research article

Melatonin reduces TNF-a induced expression of MAdCAM-1 via inhibition of NF-kB.

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Abstract

Background: Endothelial MAdCAM-1 (mucosal addressin cell adhesion molecule-1) expression is associated with the oxidant-dependent induction and progress of inflammatory bowel disease (IBD). Melatonin, a relatively safe, potent antioxidant, has shown efficacy in several chronic injury models may limit MAdCAM-1 expression and therefore have a therapeutic use in IBD.

Methods: We examined how different doses of melatonin reduced endothelial MAdCAM-1 induced by TNF-a in an *in vitro* model of lymphatic endothelium. Endothelial monolayers were pretreated with melatonin prior to, and during an exposure, to TNF-a (1 ng/ml, 24 h), and MAdCAM-1 expression measured by immunoblotting.

Results: MAdCAM-1 was induced by TNF-a. Melatonin at concentrations over 100 μm (10^{-4} M) significantly attenuated MAdCAM-1 expression and was maximal at 1 mM.

Conclusions: Our data indicate that melatonin may exert therapeutic activity in IBD through its ability to inhibit NF-kB dependent induction of MAdCAM-1.

Background

It has been previously reported that the mucosal addressin cell adhesion molecule-1 is expressed at high levels in gut associated lymphoid tissue, and that its' expression is dramatically increased during active episodes of inflammatory bowel disease (IBD), e.g. Crohns' colitis [1]. MAdCAM-1 expressed on lymphatic endothelial cells serves as a ligand for a4b7 integrin expressing lymphocytes that allows these cells to arrest and migrate within intestinal lymphatics [2–5], and appears promote development of chronic intestinal inflammatory states [1,5,6]. The role of the MAdCAM-1/a4b7 couplet in injury is well supported by studies which show that blockade of either component reduces the development of inflammation [5,6]. Therefore, therapies to diminish the net expression of MAdCAM-1 in response to the pro-inflammatory cytokines mobilized

during inflammation is an important potential avenue for research. We have previously described that several therapeutic agents which are currently used for IBD therapy (dexamethasone, IL-10) attenuate MAdCAM-1 expression and may explain part of the basis of therapy with these agents [7]. Based on these results, we wished to determine if melatonin could have a significant impact on the expression of MAdCAM-1 in lymphatic endothelial cells that have been stimulated with TNF-a, and whether TNF-a induced NF-kB activation in lymphatic endothelium is reduced by MAdCAM-1.

Methods
Reagents
Mouse TNF-a was purchased from ENDOGEN (Stoughton, MA).

Cell culture
SVEC4-10, an SV40 transformed lymphatic derived endothelial cell line which expresses MAdCAM-1 in response to TNF-a or IL-1b exposure [8] was maintained in DMEM + 10% fetal calf serum +1% antibiotic/ antimycotic. Cells were seeded at 20,000 cells/cm^2; and used immediately after reaching confluency.

Treatment protocol
SVEC 4–10 were pre-treated for 30 minutes with melatonin at 0.1, 0.5 and 1 mM, and then incubated in culture medium for 24 with 1 ng/ml TNF-a. Samples were then isolated in Laemmli sample buffer.

Western analysis of cell lysates
Western blotting was performed as described [3,7,9]. Protein concentration for loading was determined using the BCA protein assay kit (Pierce, Rockland, IL). 75 ug of protein was loaded into each lane of 7.5% SDS/PAGE gels, electrophoresed and blotted as described [9]. After electroblotting, equal protein loading was confirmed by Ponceau Red S staining. TNF-a did not alter the well-to-well protein concentration measured by protein measurement or Ponceau staining. Rat anti MadCAM-1 mAb (clone MECA367) was purchased from Pharmingen (San Diego, CA) [3]. Goat anti-rat HRP antibody (Sigma) was used as 2° Ab at a 1:2000 dilution. Blots were visualized on hyperfilm (KODAK) using enhanced chemiluminescence (ECL, Amersham Life Sciences, Piscataway, NJ). Densitometric analysis of MAdCAM-1 expression was determined using Image Pro Plus™ (Media Cybernetics, Silver Springs, MD) using a 256-shade gray scale. All experiments were repeated 3X.

Phospho-NF-kB p65 western analysis of cell lysates
To measure NF-kB activation, monolayers were either pre-treated (1 h) with melatonin, and then co-treated with TNF-a (30 min), or treated without test agents and co-

treated with TNF-a (30 min), or not treated (controls). All samples were harvested at 30 min. 75 µg of protein from each sample was separated on 7.5% SDS-PAGE gels and transferred to nitrocellulose as described. Blots were blocked with 5% milk powder in PBS + 0.1%Tween-20 at room temperature for 2 h, washed twice for 10 min with wash buffer (0.1% Tween-20 in PBS). 1° rabbit anti-phospho-NF-kB p65 polyclonal (Ser536) Ab (Cell Signaling Technology, MA) was added at a concentration of 1 µg/ml and incubated overnight at 4°C. These membranes were washed twice with wash buffer. 2° goat anti-rabbit horseradish peroxidase-conjugated secondary antibody (Sigma) was added at a 1:2000 dilution for 2 h. Lastly, membranes were washed 3 times and developed using enhanced chemiluminescence (ECL) (Amersham, La Jolla, CA). The density of phospho-NF-kB p65 staining was measured by scanning the 65 kD band, using a HP Scan-Jet™ flatbed scanner. Images were analyzed for density using Image Pro Plus™ image analysis software (Media Cybernetics, Silver Springs, MD). The data are expressed as a percentage of TNF-a-induced level of density (set as 100% or maximum).

Results
TNF-a (1 ng/ml) significantly increased the expression of MAdCAM-1 compared to untreated controls (fig. 1). At 1 ng/ml densitometry indicates that the expression of MAdCAM-1 was increased in SVEC 4–10 cells by over 5-fold at 24 hours. This represents approximately 30% of the maximal expression observed when cells are exposed to 20 ng/ml TNF-a [15]. The addition of melatonin to these cultures prior to, and during exposure to TNF-a significantly reduced the expression of MAdCAM-1. Melatonin at 100 µM, 500 µM and 1000 µM significantly reduced responsiveness to TNF-a (measured by induction of MAdCAM-1). Lower doses of melatonin (50 µM) produced slight but statistically insignificant changes in MAdCAM-1 expression. We also found that melatonin (500 µM) also significantly reduced the activation of NF-kB as measured by the phosphorylation of the NF-kB p65 subunit. TNF-a (30 min, 1 ng/ml) significantly increased phosphorylation of the p65 subunit of NF-kB (fig. 2). Pre-treatment with 500 µM melatonin significantly decreased the expression of MAdCAM-1 (compared to TNF-a alone), although this level was still slightly, but significantly elevated compared to untreated controls. TNF-a at this concentration is not wholly specific for MAdCAM-1, other adhesion molecules, e.g. VCAM-1 and ICAM-1 are also similarly mobilized by this concentration of TNF-a (data not shown), while the total cell protein is unaltered.
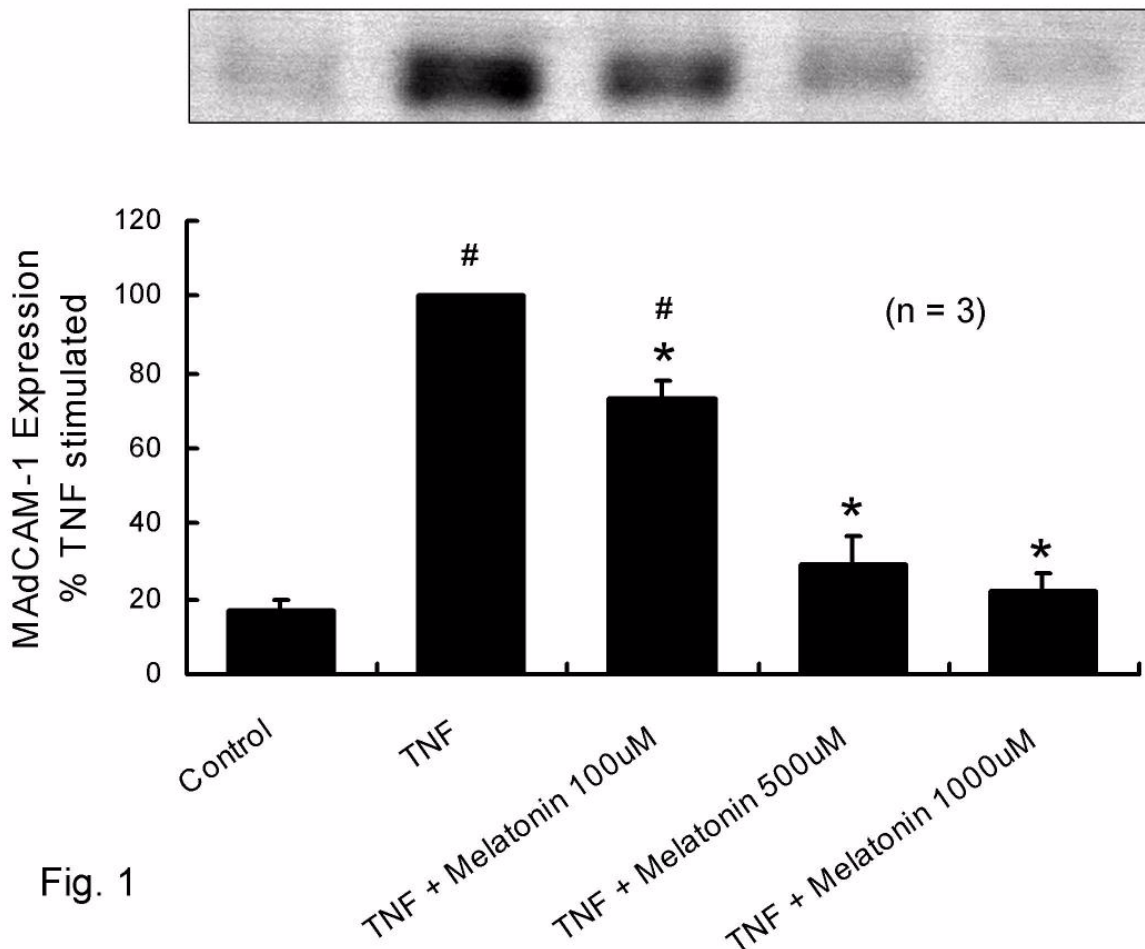
Figure 1
Melatonin significantly attenuates TNF-a induced MAdCAM-1 Expression. Figure 1 shows the induction of MAd-CAM-1 by TNF-a (1 ng/ml), and its inhibition by increasing concentrations of melatonin (0.1, 0.5 and 1 mM). * = significantly different (p < 0.05) from control.

Discussion

MAdCAM-1 is an endothelial cell surface molecule selectively expressed on 'high' endothelium that is required for lymphocyte homing to lymphatic vessels, especially to gut-associated lymphoid tissues [1,2,10–14]. Interactions mediated by MAdCAM-1 and its receptor, $\alpha 4\beta 7$, mediate lymphocyte homing to the intestine which initiates and sustains gut inflammation in inflammatory bowel disease (IBD). This notion is well-supported by studies demonstrating that antibodies against either MAdCAM-1 or the MAdCAM-1 ligand, $\beta 7$-integrin, block lymphocyte recruitment to the inflamed colon, and will reduce experimental IBD [5,6]. Therefore, interference with either MAdCAM-1 binding or the net expression of MAdCAM-1 may be beneficial in IBD therapy. Here we showed that an endogenous, relatively safe and well tolerated antioxidant, melatonin, attenuated MAdCAM-1 expression in re-

sponse to TNF-a.. The level of protection provided by 100–1000 μM melatonin was comparable to that obtained with high doses of IL-10 or dexamethasone that we described in a previous *in vitro* study [9]. It is worth noting that TNF-a does not specifically mobilize only MAdCAM-1, nor does melatonin inhibit expression of only MAdCAM-1. TNF-a will also induce other ECAMs like ICAM-1 and VCAM-1 [22], and melatonin will also inhibit the expression of ICAM-1 and P-selectin [20]. Therefore, we cannot exclude possible additional contributions made by these adhesive systems in the beneficial effect of melatonin in chronic inflammation.

Several studies support the effectiveness of antioxidants as a means to limit the excessive expression of many types of endothelial cell adhesion molecules in inflammation, including that of MAdCAM-1. Melatonin is a pineal hor-
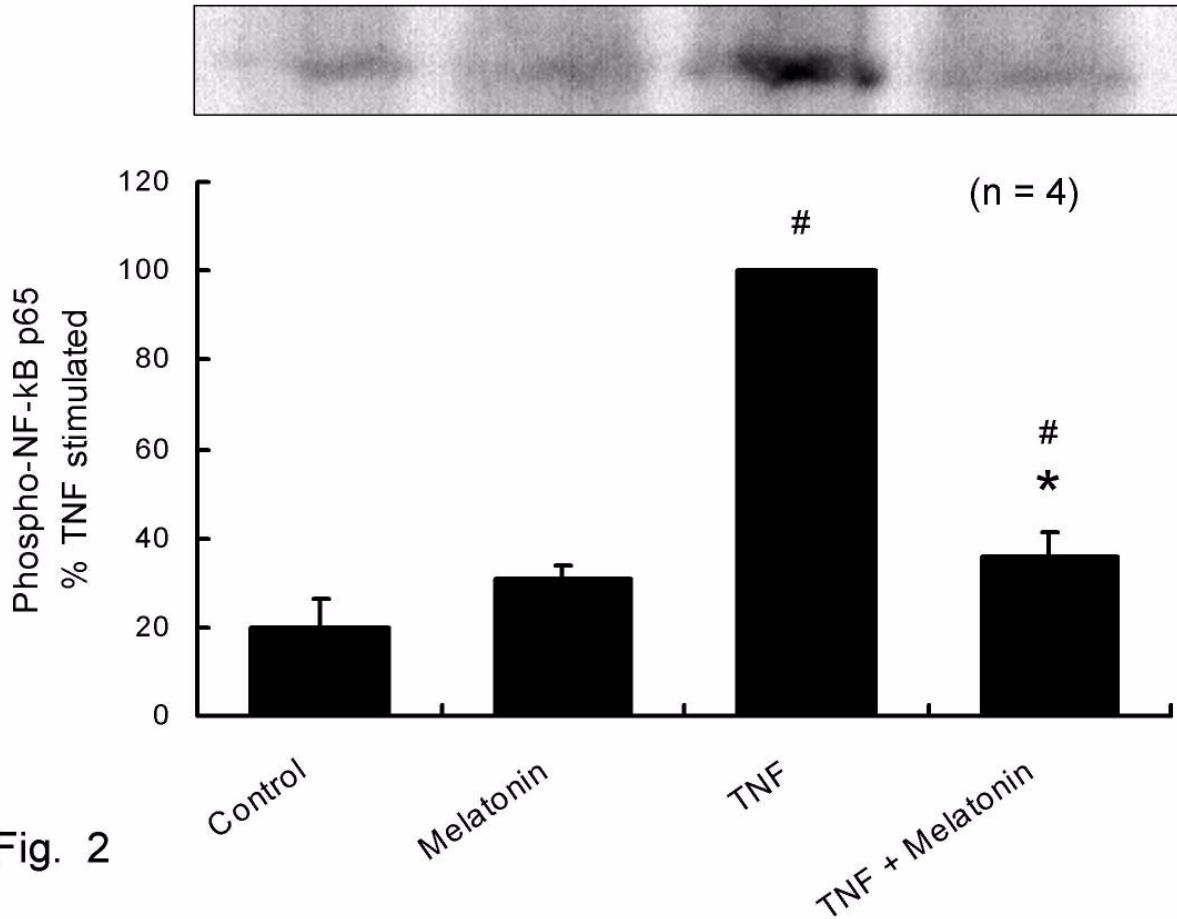
Figure 2
Melatonin prevents TNF-a induced phosphorylation of NF-kB p65. Figure 2 shows that the phosphorylation of NF-kB p65 induced by 1 ng/ml TNF-a is significantly attenuated by 0.5 mM melatonin. * = significantly different (p < 0.05) from TNF-a, # = p < 0.05 from control.

mone that has recently been proposed as a relatively safe and well-tolerated regulator of circadian rhythm [16], with strong antioxidant properties [17]. As a potent antioxidant, melatonin has been used in several chronic injury models with good success. For example, 0.7 mg/kg inhibits TNF-a induced hypotension through blockade of NO synthase activity in cancer patients [18]. In murine and rat models of colitis, melatonin also blocked several indices of gut injury in chemically induced models of colitis [19–21]. The basis of the protective effects of antioxidants in all of these models is consistent with interference with the NF-kB transcriptional system [22], which drives the expression of several genes activated during inflammation, particularly those of adhesion molecules like MAdCAM-1 [9,22]. In support of this, we have previously demonstrated that MAdCAM-1 expression is mediated at least partially through NF-kB [9]. Gilad et al. (1998) have

also demonstrated that melatonin will inhibit the NF-kB system [23].

We found here that 500 µM melatonin significantly reduced TNF-a induced activation of NF-kB, consistent with these previous findings. Melatonin also decreases the release of cytokines. Administration of melatonin significantly reduced TNF-a levels in humans with neoplasms [18]. Therefore, *in vivo*, melatonin could also prevent the development of chronic gut injury by blocking synthesis and responses to the cytokines that drive the expression of adhesion molecules like MAdCAM-1. Melatonin and melatonin binding sites are found highly concentrated within the intestine, and concentrations in the gut are between 10–100 times that found in the plasma [24]. Further, it has been suggested that the intestine is the major site for extra-pineal melatonin activity in the gut, particularly the

lymphatics [24]. Therefore, melatonin may play an important role in regulation of the intestine, but it is not yet clear whether a possible melatonin deficiency may play any role in the etiology of IBD.

Further, and importantly, although melatonin decreased the expression of MAdCAM-1 in this study, the introduction of melatonin into the treatment of human IBD should be cautiously approached. The doses of melatonin used in our study represent from 5–50 times the dose that is used as an over the counter sleep aid (3 mg). Therefore, the effects of relatively high doses of melatonin in humans should not be considered to be without the possibility for harm or toxicity. A careful evaluation of high doses of melatonin in clinical studies will be needed before it can be safely introduced into a regimen for therapy.

Conclusions
Our study shows that endothelial MAdCAM-1 expression is increased by TNF-a, and is dose-dependently inhibited by melatonin. The data suggest that melatonin may therefore be of clinical value in therapy for inflammatory bowel disease through its ability to limit the expression of adhesion molecules e.g. MAdCAM-1.

Abbreviations
MAdCAM-1 (mucosal addressin cell adhesion molecule-1), NF-κB (nuclear transcription factor κB) GALT (gut associated lymphoid tissues), TNF-a (Tumor necrosis factor alpha), SCID (severe combined immunodeficient), ICAM-1 (intercellular adhesion molecule 1), VCAM-1 (vascular cell adhesion molecule 1)

Competing interests
none declared.

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