Rebamipide inhibits free radicals derived from activated neutrophils and decreases the susceptibility of gastric mucosa to acid-induced injury by inhibiting neutrophil activation (Harada et al., 2005). Rebamipide treatment reduced inflammation in dextran sulfate sodium induced colitis by inhibiting inflammatory cytokine mediated neutrophil infiltration in the colon (Kishimoto et al., 2000).

Behcet's disease (BD) is a chronic, multi-systemic disorder with arthritic, gastrointestinal, mucocutaneous, ocular, vascular, and central nervous system involvement. This disease has a chronic course with periodic exacerbations and progressive deterioration (Shimizu, 1979). The etiology of BD is unclear, but viral infection has long been postulated as one of the main factors. Since Hulusi Behcet first proposed a viral etiology (Behcet, 1937), the viral hypothesis has been verified by detection of virus in saliva (Lee et al., 1996a), intestinal ulcers (Lee et al., 1993), and genital ulcers (Lee et al., 1996b; Bang et al., 1997a) of patients with BD. Subsequent to this finding, herpes simplex virus (HSV) inoculation of the earlobes of ICR mice resulted in the development of BD-like symptoms (Sohn et al., 1998). Manifestations in mice after HSV inoculation included multiple symptoms such as oral ulcers, genital ulcers, skin ulcers, eye symptoms, arthritic, gastrointestinal, mucocutaneous, ocular, vascular, and central nervous system involvement.
gastrointestinal ulcers, arthritis, and neural involvement, as well as skin crusting. The frequency of these symptoms was similar to that of patients with BD (Kim et al., 1988).

Abnormalities of neutrophils have been suggested to be responsible for many of the clinical manifestations of BD (Sahin et al., 1996) and serum of BD enhances superoxide production of normal neutrophils (Yoshida et al., 1998). According to the results of Matsuda et al. (2003), rebamipide improves the aphthae count and pain score in BD patients. They suggested that it may therefore be useful in the treatment and prevention of frequently recurrent oral aphthous ulcers of BD. Hahm et al. (1998), reported that combination therapy, including lansoprazole, amoxicillin, and rebamipide, against H. pylori augmented eradication rates of H. pylori with decreasing the changes of oxidative stress and cytokines. Also in Behcet’s disease, Bang (1997b) reported that combination-agent regimen is more effective than a single-agent regimen in the treatment of patients with Behçet’s disease. Colchicine is one of the most frequently prescribed medicine to the patients with Behcet’s disease (Kaklamani and Kaklamanis, 2001). In this study, we used a rebamipide combination with colchicine to reduce inflammation in BD-like mice in vivo and to reduce superoxide-producing enzyme, NADPH oxidase subunit, and to increase the effect of anti-inflammation accompanying with cytokine or chemokine expression, thereafter improving BD symptoms in mice.

2. Materials and methods

2.1. Reverse transcription PCR (RT-PCR)

Total RNA was isolated with TRIzol (Life Technologies, Helgerman, CT), according to the manufacturer’s recommendations. An amount of 2 μg of total RNA from spleens was used as a template for cDNA synthesis with SuperScript III First-Strand Synthesis System for RT-PCR kit (Invitrogen, Carlsbad, CA). The cDNA was amplified by PCR with the following primers: Amplified PCR products were visualized on 1.2% agarose gels.

| β-actin    | 5′-TGGAATCCTGTGGCATCCATGAAAC-3′ | 5′-TAAAACGCAGCTCAGTAACAGTCCG-3′ |
| IFN-γ     | 5′-AGCGGCTGACTGAACTCAGATTGTAGCTTGTACCTTTACTTCACTG-3′ | 5′-GTCAAGTTTTGGCCTGTATAGGG-3′ |
| TNFα      | 5′-GCCAGGTCTACTTTGGAGTCATTGC-3′ | 5′-ACATTCGAGGCTCCAGTGAATTCGG-3′ |
| MIP1α     | 5′- TTCTGTTGTCGGACAAGCTCCATTCTCT-3′ | 5′-TGAGAAACGTGTCCTGAATGCTTTC-3′ |
| Fas        | 5′-CTCAAGGGTACCTATAGCACTCTCCGA-3′ | 5′-CAGTGAGAAGCTGGTTCCTAGTG-3′ |
| Perforin   | 5′-ACGTGAGAAGAAGCTACATAGGC-3′ | 5′-CATAAACGTGTCGGCATAGG-3′ |
| p22phox   | 5′- TCTGTTGTCGGACAAGCTCCATTCTCT-3′ | 5′-TGAGAAACGTGTCCTGAATGCTTTC-3′ |
| p40phox   | 5′- AGCTGAGAAGAAGCTTCTGTTCG-3′ | 5′- AGACCTGACATACATAGGC-3′ |
| p47phox   | 5′- GTTGAGAAGAAGCGAGACAGCGG-3′ | 5′- GTGGATGCTCTGTGCGTTG-3′ |
| p67phox   | 5′- CTAGTGGCTGTCGGTGGCG-3′ | 5′- CACAAAGCCAAACAATCGG-3′ |
| Gp91phox  | 5′- GCAAGCCGACCACCACACAA-3′ | 5′- CCCCTCCGTCGGTCCCAA-3′ |

2.2. Real time PCR

For real time SYBR Green RT-PCR, the 20 μl reaction contained 10 μl of 2× Quantitect SYBR Green Master Mix (Qiagen, Valencia, CA, USA)
containing a hot start Taq polymerase, 0.4 μl mix of 2 reverse transcriptases, 0.5 μl (10 ng/μl) of template and 0.8 μl of primers (forward: CTGATGGGAGGAGATGTCTA, reverse: GTTATTTGTCATTC-CGGTGT). The ABI 7900 HT thermal Cycler (Lab Centraal B.V., Haarlem, The Netherlands) was used for all real time RT-PCR assays. Reverse transcription was carried out at 50°C for 30 min, followed by denaturation at 95°C for 15 min. DNA was amplified with 40 PCR cycles at 95°C (30 s), 55°C (30 s), and 72°C (30 s). Real time RT-PCR data was collected for 15 s at 75°C to avoid non-specific fluorescence due to primer dimers occurring at low template concentrations. For generation of standard quantitation curves, the cycle thresholds values were plotted proportionally to the logarithm of the input copy numbers. Negative controls were included in each run.

2.3. Animal experiments

Male ICR mice (4 to 5 week old) were infected with HSV-1 (F strain) grown in Vero cells, as previously described (Sahin et al., 1996). Virus inoculation was performed twice, with a 10 day interval, followed by 16 weeks of observation. Mice were bred in temperature and light controlled, conventional rooms (20–22 °C, 12 h light cycle starting at 8:00 a.m.). The mice had free access to food and water. During the experimental period, the animals were closely observed and photographed. Animals were handled in accordance with a protocol approved by the animal care committee of Ajou University School of Medicine. For each mouse, 200 μl artificial gastric fluid or 2 μg colchicine in 200 μl gastric fluid or 150 μg rebamipide in 200 μl gastric fluid or 2 μg colchicine in 200 μl gastric fluid plus 150 μg rebamipide was treated orally once per day. Treatment was done for 5 consecutive days. Two hour or 20 days after last administration, spleens were isolated for RT-PCR and real time PCR, and serum was collected for ELISA. Schematic diagram of in vivo treatment period in BD mice is in Fig. 1.

2.4. BD symptoms

Manifestations in mice after HSV inoculation involved multiple symptoms, which included oral ulcers, genital ulcers, skin ulcers, eye symptoms, gastrointestinal ulcers, arthritis, and neural involvement, as well as skin crust. Oral, genital, other skin ulcers (including bulla and crust) and eye symptoms were all classified as major symptoms. The other symptoms were classified as minor symptoms. Of the total number of HSV-injected mice, 14% developed to BD mice. The disappearance of symptoms and decrease in lesion size constituted improvement as like that in patients. The time interval of observation of the animals was once per week after HSV inoculation. The Severity score of BD was followed by determination of the value of Behcet’s disease activity index, as outlined in the BD Current Activity Form 2006 (www.behcet.ws/pdf/BehcetsDiseaseActivityForm.pdf). Among the patients’ symptoms, mouth ulceration, genital ulceration, erythema, skin pustules, skin ulceration, joints-arthritis, diarrhea, red eye (right, left), reduced vision (right, left), loss of balance, discoloration, and swelling of the face were selected and analyzed in the BD mouse model. The score of each symptom is one and when the score was added up, the total was used in determining the Severity score of BD. Before and after treatment, the Severity score was measured and compared. Mice exhibiting symptoms were photographed to document improvement after treatment, with significantly reduced symptoms.

2.5. ELISA

Serum was analyzed using commercial ELISA kits for the detection of mouse TNFα (R&D Systems Inc., Minneapolis, MN). The ELISAs were carried out according to the manufacturer’s instructions. Means and standard deviations were calculated using ELISA values determined for each well. The ELISA reader was a Bio-Rad model 680 microplate reader, and wavelength was 450 nm.

2.6. Statistical analysis

All data are represented as the mean±SE. Statistical differences between the experimental groups were determined using the Qui square test, Student’s t test, and Bonferroni correction.

3. Results

3.1. Combination therapy attenuated the symptoms of mice and decreased the Severity score

At 2 h after administration, combination treatment (rebamipide plus colchicine) decreased BD symptoms such as skin ulcer, genital ulcer, and arthritis in 6 out of 8 BD mice. But in 2 out of 8 BD mice, the Severity score was increased and symptoms were deteriorated. Combination treatment or colchicine treatment could not completely prevent the development of this disease in this model.

The Severity score, in combination treatment, was increased in 1 of 6 mice. At 20 days after administration, combination treatment improved BD symptoms in 5 out of 6 mice. Colchicine treatment improved in 4 of 5 mice. The Severity score, before and after treatment, was calculated as outlined in the BD Current Activity Form 2006. At 20 days after administration, combination treatment decreased 23.5% of the Severity score compared to before administration. In contrast, colchicine treatment decreased 14.3% of the Severity score compared to before administration. Combination treatment decreased more than colchicine treatment as 9.2% (Fig. 2).
Therefore, combination treatment was more efficient to decrease the Severity score than colchicine treatment though this difference was not statistically significant.

3.2. RT-PCR reveals the difference of expression according to treatment

Effect of combination treatment in spleens of BD mice was determined by RT-PCR. Two hours after last administration, in the combination treated group, Fas, p40 phox, and gp91 phox mRNA expression were lower than to rebamipide treated or colchicine treated group. Twenty days after last administration, in the combination treated group, TNFα, MIP-1α (macrophage inflammatory protein-1 alpha), p40 phox, p47 phox, p57 phox, and gp91 phox mRNA expression was lower than rebamipide treated or colchicine treated group by reverse transcriptase PCR (RT-PCR) (Fig. 3).

3.3. Combination therapy decreased the expression of NADPH oxidase subunit by real time PCR

The effects of combination treatment on the NADPH oxidase subunits, p22 phox, p40 phox, p47 phox, p67 phox, and gp91 phox, in spleens of BD mice were determined by real time PCR. At 2 h after last administration, in the combination treated group, p40 phox, p47 phox, p67 phox, and gp91 phox mRNA was markedly downregulated compared to the colchicine treated group (Fig. 4A). In these four NADPH oxidase subunits, the combination treated group was markedly downregulated compared to the colchicine treated group, and statistically different. After 20 days of the last administration, in the combination treated group, p40 phox and p47 phox were downregulated compared to the colchicine treated group and was statistically significant (Fig. 4B).

3.4. Combination therapy could influence the serum level of TNFα in BD mice

To compare the serum level of TNFα between combination treatment and colchicine treatment, the serum was collected from each group at 2 h and at 20 days after 5 consecutive oral administrations. As determined by ELISA, the TNFα expression levels were as follows: 36.87±25.63 pg/ml for the combination treatment and 46.04±39.67 pg/ml for the colchicine treatment in the 2 hour group; 25.29±35.33 pg/ml for the combination treatment and 38.64±57.50 pg/ml for the colchicine treatment in the 20 days group. TNFα expression levels were 20% lower for the combination treatment compared to the colchicine treatment in the 2 hour group, and 35% lower for the combination treatment compared to the colchicine treatment in the 20 days group. But this difference was not statistically significant (Fig. 5).

4. Discussion

The present study has demonstrated that combination treatment with rebamipide and colchicine, decreased the mRNA level of NADPH oxidase subunits, serum level of TNFα, and the Severity score of this disease compared to colchicine treatment alone. The serum level of TNFα and the Severity score of this disease between these two groups were not significantly different. The mRNA levels of inflammation related cytokine, IFNγ and TNFα, chemokine, MIP-1α, cell death related molecules, perforin and Fas, reactive oxygen species producing enzyme NADPH oxidase subunits, p40 phox, p22 phox, gp91 phox, p47 phox, and p67 phox were lower in combination treatment compared to colchicine treatment by RT-PCR in spleen tissue of BD mice. IFNγ and TNFα are the key cytokine produced by Th1 cells and is an important immunoregulatory factor (Trinchieri and Perussia, 1985). IFNγ and TNFα, as a possible proinflammatory cytokines towards the T helper type 1 has been suggested (Emmi et al., 1997). The chemokine MIP-1α has also been reported to be differently regulated in normal and BD-like mice (Lee et al., 2004). Perforin is related to cell apoptosis. Perforin dependent pathway is a major killing system when cytotoxic T lymphocytes induce their target-cell death (Ono et al., 2002). Fas is a cell-surface protein that belongs to the tumor necrosis factor receptor family. Fas system dysregulation leads to uncontrolled lymphoproliferation, indicating that the Fas/Fasl system plays a central role in immune reaction control via cell death activation (Chervonsky et al., 1997; Nishimura et al., 1997). NADPH oxidase contributes directly to oxidative stress (Tammariello et al., 2000) by producing increased levels of ROS (Martins Chaves et al., 2002). It has also been documented that ROS production is detected in increased expression of oxidative stress and increased ROS production and oxidative stress propagation contribute to chronic inflammation (Afonso et al., 2007).

In this study, the mRNA expression of NADPH oxidase subunits in BD mice were lowered by treatment with rebamipide and colchicine combination. Rebamipide acts as a free radical scavenger and as an inhibitor of NADPH oxidase (Naito et al., 1995). Real time PCR also reveals that the expression of p40 phox, p47 phox, p67 phox, and gp91 phox was lower in combination treatment. The decrease of these subunits connected to the synthesis of NADPH oxidase, therefore, lower synthesis of the NADPH oxidase produced less amount of reactive oxygen species. Down regulated reactive oxygen species related to decrease the triggering of the inflammatory response (Yao et al., 2007). The Severity score was 23.5% lower after the combination treatment than before the treatment, compared to 14.3% lower in the colchicine treatment. Rebamipide has been reported to possess antiulcer properties against ethanol or acid-induced gastric mucosal damage. These effects have been interpreted by its pharmacological action on prostaglandin synthesis in gastric epithelium (Yamasaki et al., 1987). Rebamipide also has antiulcer properties against herpes...
simplex virus-induced mucocutaneous ulcer and arthritis and this effect could be interpreted by its pharmacological action on decreasing the synthesis of NADPH oxidase subunits. Rebamipide may suppress active oxidant production through modulating the activation of NADPH oxidase (Ono et al., 2002).

In conclusion, our results demonstrate that rebamipide helped the function of colchicine to improve the HSV induced BD symptoms by inhibiting the expression of NADPH oxidase in a vivo mouse model. This suggests that rebamipide may protect against HSV induced inflammation by lowering the level NADPH oxidase and TNFα. However, the difference of the serum level of TNFα and the Severity score were statistically not different. Further studies are required to define the exact action of rebamipide as a therapeutic agent for improving inflammation of BD.

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References
