Impaired Interleukin-2 Production in Active Ulcerative Colitis is Reversed by Calcium Ionophore Plus Phorbol Myristate Acetate and Related to Altered Intracellular Ca²⁺ Responses

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Phytohemagglutinin (PHA)-induced IL-2 production in vitro by peripheral blood mononuclear cells (PBMC) and lamina propria mononuclear cells (LPMC) from patients with active UC (n=24, n=8, respectively) was significantly less than that in controls (n=13, n=8, respectively) and inactive patients (n=11). In contrast, PBMC from inactive disease showed no significant difference when compared with the controls. Depressed IL-2 production in active UC was not reversed by the addition of anti-CD3 monoclonal antibody plus phorbol myristate acetate (PMA), but was largely reversed by adding calcium ionophore plus PMA. Using a fluorescent Ca²⁺ probe fura-2, we found that after PHA stimulation LPMC from patients with active UC showed a lower magnitude of rise in intracellular free calcium concentration ([Ca²⁺]ₐ) than control cells. These results suggest that impaired PHA-induced IL-2 production in active UC may be related to some alterations of the early signaling events that cause elevation of the [Ca²⁺]ₐ.

Introduction

Previous studies have shown that interleukin-2 (IL-2) production is decreased in vitro in patients with active ulcerative colitis (UC) compared with controls (1, 2). IL-2 is a cytokine produced by T cells in response to mitogen or antigen stimulation, and it plays a crucial role in the regulation of immune responses (3, 4). Thus, deficient IL-2 activity can lead to immunologic abnormalities in UC patients, such as the disruption of normal T cell proliferation and differentiation. T cell activation during antigen recognition is mediated by at least two synergistic intracellular signals; i.e., an increase in the intracellular free calcium concentration ([Ca²⁺]ₐ) and the activation of protein kinase C (PKC) (5, 6). However, the combination of a calcium ionophore and a PKC activator such as phorbol myristate acetate (PMA) bypasses the requirement for an antigen-induced signal at the onset of T cell activation (7). The purpose of this study was to investigate the IL-2 activity generated in response to phytohemagglutinin (PHA) or PMA combined with anti-CD3 monoclonal antibody (mAb) or calcium ionophore using peripheral blood mononuclear cells (PBMC) and lamina propria mononuclear cells (LPMC) obtained from patients with UC. We also evaluated intracellular Ca²⁺ mobilization under these conditions in an attempt to clarify the mechanism of the decreased PHA-induced IL-2 production in active UC.

Materials and Methods

Subjects

Biopsy specimens (n=8) and peripheral blood (active disease: n=24, inactive disease: n=11) were obtained from 24 patients with UC who were undergoing diagnostic or follow-up colonoscopy at Nagoya University Hospital. The group included 14 males and 10 females, ranging in age from 19 to 59 years (mean age: 36.5±5.8 years). The diagnosis of UC was confirmed by clinical, endoscopic, radiologic, and histological criteria (8, 9). Fourteen patients had total colitis and 10 had left-sided colitis. The duration of disease ranged from 1 month to 12 years. At the time of the study, 9 patients were receiving corticosteroids alone, 3 were on sulfasalazine alone, and 12 were being treated with both agents. None of them were receiving calcium channel blockers or cyclosporin A. To isolate control non-UC LPMC, large bowel specimens were collected...
from 8 patients undergoing colectomy for colon cancer (n=6) or colonic diverticular disease (n=2). Five of these patients were men and three were women, ranging from 41 to 68 years of age (mean age; 58.6±6.5 years). Peripheral blood samples were also obtained from 13 healthy volunteers (8 men and 5 women) who ranged in age from 28 to 45 years (mean age; 39.8±4.5 years) as controls. All tissue and peripheral blood specimens were obtained after informed consent was given in accordance with the Helsinki Declaration.

Isolation of peripheral blood mononuclear cells
PBMC were isolated from 30 ml of heparinized peripheral blood by centrifugation on a Ficoll-Hypaque gradient (Pharmacia Fine Chemicals, Piscataway, NJ). After washing twice with Hank’s balanced salt solution (HBSS, Gibco, Grand Island, NY), the cells were resuspended at a concentration of 1x10^6/ml in RPMI 1640 medium (Gibco) supplemented with L-glutamine, HEPES buffer, penicillin, streptomycin, and 10% heat-inactivated fetal calf serum (FCS).

Isolation of lamina propria mononuclear cells
Colonoscopic biopsy specimens were obtained from the actively involved part of the bowels (rectum or sigmoid colon) of patients with UC. Control mucosae were taken from histologically normal portions of the surgical specimens, at least 10 cm away from the sites affected by the disease. The method used to isolate LPMC was a modification of the original technique of Bull and Bookman (10), and is described in detail elsewhere (11). Briefly, colonoscopic biopsy specimens or colonic mucosae obtained by a biopsy forceps from surgical specimens were washed free of mucus with HBSS, after which the tissues were stirred for 30 minutes in HBSS with 1 mM dithiothreitol and then twice for 90 min with 1 mM ethylenediamine tetraacetate to remove epithelial cells. The tissues were then gently stirred for 12 hours at 37°C in HBSS with 0.02% collagenase (Worthington Diagnostic Systems Inc., Freehold, NJ) and 0.01% deoxyribonuclease (Worthington). Subsequently, the cell suspension was filtered through nylon mesh (100 μm), and the resultant LPMC were purified on a Ficoll-Hypaque gradient, washed twice, then resuspended in the culture medium.

Induction of interleukin-2 production by peripheral blood and lamina propria mononuclear cells
For the induction of IL-2 production, cells were cultured at 1x10^6/ml in a humidified atmosphere of 5% CO2; 95% air for 48 hours at 37°C in the culture medium under the following conditions: (a) with 10 μg/ml PHA (Difco Laboratories, Detroit, Mich.) (12), and (b) with 40 ng/ml phorbol-12-myristate-13-acetate (PMA; Sigma Chemical, St. Louis, Mo.) plus 1:100 dilution of anti-CD3 mAb OKT3 (Ortho Diagnostic System Inc., Raritan, NJ). This particular combination of PMA and OKT3 is known to induce IL-2 messenger RNA in T lymphocytes (13). In addition, (c) a combination of 40 ng/ml PMA and calcium ionophore A23187 (Calbiochem, La Jolla, CA) at 250 nM was used. The optimal dosages of these agents and the appropriate incubation period for IL-2 production were determined by the previous study (2). At the end of culture, cells were pelleted by centrifugation and the supernatants were collected, sterilized by filtration (0.45 μm), and stored at -70°C until assayed for IL-2 activity.

Assay of interleukin-2 activity
The IL-2 activity of the culture supernatants was measured by bioassay using an IL-2-dependent cytotoxic mouse T-cell line (CTL-N, kindly provided by Dr. Fiocchi, Case Western Reserve University, Cleveland, OH). Serial dilutions of culture supernatant were added to CTL-N cells in round-bottomed microtiter plates (1x10^3/well) and incubated for 18 hours. The cultures were then pulsed for 6 hours with 0.5 μCi of [3H-thymidine (New England Nuclear, Boston, MA) and harvested onto glass fiber filters, after which the radioactivity was determined using a liquid scintillation counter (Beckman, Palo Alto, CA). One unit of IL-2 activity was defined as the inverse of the sample dilution yielding the same number of counts per minute response of a standard recombinant IL-2 (kindly provided by Takeda Chemical Ind., Osaka, Japan), as determined by probit analysis (14).

Measurement of the intracellular Ca2+ responses using fura-2
Intracellular calcium responses were assessed by monitoring the fluorescence of mononuclear cells loaded with the calcium indicator fura-2 (15, 16). For the dye loading, LPMC (5x10^6/ml) isolated from active UC patients and controls were incubated with fura-2 acetoxymethyl ester (4 μM, Dojin Chemical Co., Kumamoto, Japan) in Ca2+/Mg2+-free PBS for 30 min at 37°C. The cells were then washed twice and resuspended at 1x10^6/ml in Ca2+/Mg2+-free PBS containing 10 mM HEPES, 0.5% FCS, and 10 mM glucose (pH 7.4). Aliquots of cells were dispensed into quartz cuvettes and the external Ca2+ concentration was adjusted to 1 mM with CaCl2. Changes in fluorescence were monitored using a fluorescence spectrophotometer (CAF-100, Japan Spectroscopy Co. Ltd.) after stimulation with either PHA (10 μg/ml), OKT3 (diluted 1:100) plus PMA (40 ng/ml), or ionomycin (250 nM, Sigma Chemical Co., St. Louis) plus PMA. During measurement, the temperature of the cell suspension was kept at 37°C and the suspension was continuously stirred. The excitation and emission wave-lengths were 340/ 380 and 510 nm, respectively. Changes of [Ca2+]i were expressed as the ratio of the two fluorescence readings.

Statistical analysis
Statistical analysis was performed using Kruskal-Wallis test followed by Wilcoxon’s rank sum test. Probability values less than 0.05 were considered to indicate significance. All data were expressed as the mean±SEM.

Results
Interleukin-2 production by PBMC
In healthy volunteers, PHA-induced IL-2 production by PBMC averaged 35.6±5.5 U/ml. The values from UC patients...
were 3.9±0.8 U/ml for active disease and 33.0±19.4 U/ml for inactive disease (Fig. 1). PHA-induced IL-2 production by PBMC from patients with UC was significantly depressed in active phase, but not in quiescent disease when compared with controls (p<0.01). In contrast, IL-2 production by PBMC stimulated with A23187 plus PMA showed no significant difference between the controls (285.7±57.6 U/ml) and active UC patients (258.4±88.6 U/ml) (Fig. 2).

**Interleukin-2 production by LPMC**

Eight pieces of tissue samples obtained from each patient by an endoscopic biopsy forceps offered 1.1±0.4x10^6 cells/100 mg wet weight after enzymatic treatment and purification by a Ficoll-Hypaque gradient. In contrast, the cell yield from surgical specimens after the same procedure was 0.6±0.1x10^6 cells/100 mg wet weight. The mean viability of cells was 93.2±1.8% and 91.4±1.9%, respectively. IL-2 production by PHA-stimulated LPMC was 6.8±4.2 U/ml in patients with active UC and 29.7±8.2 U/ml in controls. IL-2 production by LPMC stimulated with a combination of OKT3 plus PMA or A23187 plus PMA were also investigated. In control experiments, IL-2 production was increased after the addition of PMA in combination with OKT3 or A23187 (56.5±28.2 versus 120.1±34.2 U/ml, respectively). On the other hand, IL-2 production by LPMC from UC patients was not increased by stimulation with OKT3 plus PMA (5.7±3.2 U/ml), but was significantly (p<0.01) increased and yielded a comparable level of IL-2 activity (95.2±39.8 U/ml) after stimulation with A23187 plus PMA (Fig. 3).
Fig. 4. Representative changes of the intracellular Ca\textsuperscript{2+} level in LPMC from a control (A, B, C) and a patient with active UC (D, E, F) induced by the stimulation with PHA, OKT3 plus PMA, and ionomycin plus PMA, respectively.

Changes of intracellular Ca\textsuperscript{2+} levels induced by PHA, OKT3 plus PMA or a calcium ionophore

After LPMC from controls and patients with active UC were loaded with fura-2 acetoxyethyl ester, the cells were stimulated with PHA, OKT3 plus PMA, or ionomycin plus PMA. In control LPMC, PHA induced an immediate increase in [Ca\textsuperscript{2+}], and OKT3 plus PMA or the combination of ionomycin plus PMA caused further elevation of [Ca\textsuperscript{2+}], which reached a maximal value after two minutes (Fig. 4A, B, C). In contrast, in LPMC from patients with active UC, PHA or the combination of OKT3 plus PMA triggered smaller increases in [Ca\textsuperscript{2+}] than in controls. On the other hand, ionomycin plus PMA induced a long-lasting increase in [Ca\textsuperscript{2+}] which was comparable to that in controls (Fig. 4D, E, F). The magnitude of intracellular Ca\textsuperscript{2+} response to PHA in control was significantly larger than that observed in patients with active UC (Fig. 5).

Discussion

This study showed that PHA-induced IL-2 production by LPMC and PBMC was reduced in patients with active UC. Previous investigations using PBMC and LPMC isolated from surgical specimens have also indicated the impairment of mitogen-induced IL-2 production in UC patients (2, 17). The precise mechanism of this abnormality, which is also recognized in some autoimmune diseases such as systemic lupus erythematosus (SLE) or rheumatoid arthritis (18, 19), is still unclear. Several investigators have indicated that IL-2 hyposecretion under these conditions may be due to the refractoriness of T cells that have been continuously activated in vivo (20, 21). Increased in vivo activation of T cells might
result in defective IL-2 production in response to further stimulation in vitro. Another possibility has been suggested that an intrinsic defect of IL-2 production might reside in the dysfunc-
tion of SLE lymphocytes (22, 23) or accessory cells (24). In
patients with SLE there were severe defects in both IL-1 and IL-
2 production by PBMC. It was reported that low IL-2 production
in SLE could not be restored to normal by adding purified
IL-1 in some patients, nor by removal of monocytes, and
speculated that there existed possible multifactorial mech-
isms (25).

On the other hand, the impaired ability of UC T cells to
produce IL-2 may be related to one or more abnormalities in
signal transduction, inadequate expression or function of
receptors for cytokines, a block in the pathway from T cell
receptor (TCR) to PKC activation or intracellular Ca2+
mobilization, or an absence of appropriate costimulatory signals (26). Recently, Matsuura et al showed that both IL-2Rα and IL-2Rβ mRNA were abundant among mucosal cells and suggested that the defect of IL-2 production in UC may be related to decreased
IL-2 transcription on the basis of a Northern blot analysis in the
experiments using PBMC and LPMC (27). Though TCR-
elicited signals result in the activation of the factors that
regulate cytokine promoter activity are as yet only partially
understood, this signal transduction pathway induces several
nuclear complexes which are known to regulate cytokine gene
expression, including NK-κB, AP-1, AP-3, NF-AT and octamer-
binding factors (28). Besides these nuclear factors, the regulation
of the mRNA half-life or stability and the rate of transcription
may be responsible for decreased IL-2 transcription.

In the present study, we explored whether normalization of
IL-2 production could occur in vitro when several activation
agents were applied to stimulate T cells from active UC pa-
tients. In particular, we focused on the response mediated via
the CD3/TCR complex and demonstrated that depressed IL-2
production capacity of LPMC from active UC patients could be
restored by PMA in combination with calcium ionophore
A23187, but not by PMA combined with an anti-CD3 mAb. T
cell activation by ligands that interact with the CD3/TCR
complex (e.g., anti-CD3 mAb) requires the presence of
monocytes. Although PHA induces polyphosphoinositide turno-
ver (29), PKC activation is also necessary for optimal T cell
activation and cytokine production. Because PMA is consid-
ered as a natural analogue of diacylglycerol which binds to and
activates PKC, PMA can be used to substitute for this function
of monocytes and bypass the signal transduction pathway when
administered in combination with a calcium ionophore. Addi-
tionally, unlike with SLE, there is no evidence of decreased
production of IL-1 in patients with UC (30). Thus, these results
suggest that the mechanism of PHA-induced in vitro IL-2
hyposecretion by PBMC and LPMC from UC patients might be
related to an alteration of the early events during signal
transduction through the CD3/TCR complex on T cells.

Next, we examined the changes in intracellular Ca2+ levels
induced by PHA, OKT3 plus PMA, or ionomycin plus PMA.
The intracellular Ca2+ response to PHA was reduced in amplitu-
de in LPMC from most of the active UC patients. In contrast,
Kuroiwa et al


