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Tea Polyphenol Inhibits Allostimulation in Mixed Lymphocyte Culture

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Green tea polyphenols are known to protect allogenic donor tissues from acute rejection by their recipients. This immunosuppressive effect may be generated by a unique chemical property of the major component, epigallocatechin-*o*-gallate (EGCG), which can block specific cell surface molecules of the donor tissues. To test this hypothesis, we examined the effects of EGCG on the murine mixed lymphocyte reactions. EGCG treatment of stimulator cells significantly attenuated the proliferation of responder T cells. The proliferation did not recover upon the secondary stimulations by fresh untreated cells or exogenous IL-2. Flow cytometric analyses showed that EGCG treatment decreased the staining intensities of various cell surface molecules including MHC II, which plays a major role in antigen presentation, and B7.1, B7.2, and their ligand, CD28, which are required for costimulatory signals in T-cell activation. These results suggest that an anergic state of alloreactive T cells may be induced by either weakening of antigen signaling or blockage of costimulatory signals with EGCG. Other possible mechanisms behind the immunosuppressive effect and a potential use of EGCG treatment of donor tissues in transplantation medicine are discussed.

Key words: Costimulatory signals; Allorecognition; Polyphenol; EGCG

INTRODUCTION

Studies on transplantation immunology have shown that some plant-derived chemicals can prevent transplantation-associated problems such as graft rejection and graft versus host disease (GVHD) (5,19,21). Among these chemicals, green tea polyphenols, better known as anti-cell proliferation agents, were recently found to prevent allogenic rejection of nerve transplants in rats, where immersing peripheral nerve bundles in a polyphenol solution before transplantation completely avoided rejection by the host (7). This process should involve immunological interactions between the polyphenol-treated donor tissue and the host immune cells but not a direct proliferation arrest of the latter, which is not treated with polyphenols.

The exact mechanism of this immunosuppressive effect and a specific active component of the polyphenols have not yet been defined (21). Because the polyphenols are known to bind to cell surface macromolecules (12) and impair receptor–ligand interactions (2), we hypothesized that they would attach to such cell surface molecules that are involved in antigen recognition and interfere with the recognition of a donor tissue as foreign by its recipient. In the present study, we tested this hypothesis by examining the influence of (−)-epigallocatechin-*o*-gallate (EGCG), a major green tea polyphenol component, on various parameters of allostimulation using a murine mixed lymphocyte culture (MLR) system.

MATERIALS AND METHODS

Mice

Female BALB/c and C57Bl/6 mice were purchased from Japan SLC (Shizuoka, Japan) and housed under specific pathogen-free conditions. Mice were used at the age of 9–11 weeks old. All experiments were approved by the local review board of Kyoto University and were conducted in accordance with the national and international guidelines for laboratory animal care.

EGCG Treatment Protocol

Spleens were aseptically removed from BALB/c (H-2K^d) and C57Bl/6 (H-2K^b) female mice, and cell suspen-

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sions were prepared by teasing and flushing the spleens with forceps and filtering through a 50-μm filter in Hank's balanced salt solution (HBSS) containing 1% FCS. Cells were centrifuged at $300 \times g$ for 5 min, resuspended in 10 ml RPMI-1640 (Sigma-Aldrich, Tokyo, Japan) containing 0, 200, or 400 μM EGCG/10^7 cells, and incubated at 4°C for 1 h. After incubation, cells were immediately washed twice with RPMI-1640 (10% FCS) before being used in mixed lymphocyte reactions or cell surface marker analyses.

Mixed Lymphocyte Reaction (MLR)

Splenocytes derived from BALB/c mice were used as "stimulator" cells and those from C57Bl/6 as "responder" cells. In both one-way and two-way MLRs, the stimulator cells were untreated or treated with EGCG before coculture. In a one-way MLR, cells from BALB/c mice were treated with 20 ppm mitomycin C (MMC) (MP Biomedicals, Aurora, OH) in RPMI-1640 supplemented with 10% FCS at 37°C for 30 min to arrest their proliferation before EGCG treatment. In a two-way MLR, both stimulator and responder populations were left capable of responding. Each cell population was resuspended in complete medium (RPMI-1640 supplemented with 10% FCS, 100 U/ml penicillin, and 100 ppm streptomycin) to a final concentration of 5.0×10^6 cells/ml. The stimulator and responder cells were mixed at a 1:1 ratio (100 μl each) in 96-well microplates and incubated triplicate at 37°C with 5% CO_2 for 72 h. Cell proliferation was assessed by counting an aliquot of harvested cells from each MLR well with a trypan-blue exclusion method.

Measurement of IL-2

After the period of MLR incubation, supernatants from the cultures were assayed for IL-2 production using cytokine quantification ELISA kits according to manufacturer's instruction (eBioscience, San Diego, CA). The detection limit of IL-2 was 10 pg/ml.

Secondary Stimulation of MLR Cultures

One-way MLR wells were separately prepared, and after 48 h of incubation the cells were recultured with MMC-treated fresh BALB/c splenocytes (5.0×10^6 cells/ml) in complete medium. Some MLR wells were cultured with exogenous recombinant murine IL-2 (1.7 IU/ml) (eBioscience). These MLR cultures were incubated for an additional 48 h, and cell proliferation was assessed as described earlier.

Flow Cytometric Analyses of Cell Surface Molecules

Flow cytometric analyses of cell surface molecules of the control and EGCG-treated splenocytes were performed using a series of fluorescence-labeled monoclonal anti-mouse CD/MHC antibodies and corresponding isotype-matched control antibodies. These antibodies are to detect CD28, CD49d, B7.1 (CD80), B7.2 (CD86), MHC I, MHC II (eBioscience), and TCRαβ (Pharmingen, San Diego, CA). The splenocytes were treated with red blood cell lysis buffer (eBioscience) for 5 min, and mononuclear cells were treated with EGCG-containing media and washed with RPMI (10% FCS) and a staining buffer (eBioscience) at 4°C. Each splenocyte sample was reconstituted in 50 μl of the staining buffer and subjected to antibody labeling for 15 min. After washing the samples twice with the staining buffer, a minimum of 10,000 events per sample was collected and analyzed on a FACScan flow cytometer (Becton Dickinson, Franklin Lakes, NJ).

Detection of Cell Proliferation by Flow Cytometry

To examine if the proliferation of responder T cells was attenuated by coculturing with EGCG-treated stimulator cells, cell samples from MLR cultures after 72 h were stained with FITC-labeled anti-mouse CD71 monoclonal antibody (BD Pharmingen) and with PE-labeled anti-mouse Thy 1.2 monoclonal antibody (Caltag Laboratories, Burlingame, CA) and analyzed on FACScan.

Detection of Apoptosis by Flow Cytometry

To examine whether apoptosis is induced in the responder and EGCG-treated stimulator cells in MLR culture, apoptosis was examined by flow cytometry. Cells collected from MLR plates after 24 h of incubation were washed once with PBS. The cells were stained with PE-labeled anti-mouse H-$2K^b$ monoclonal antibody (Caltag Laboratories, CA), which stains the responder cells (C57Bl/6). Then apoptotic cells were stained with FITC-labeled Annexin V according to the manufacturer's instructions (R&D Systems, MN) and analyzed on FACScan.

Statistical Analysis

All results are expressed as mean ± SD. Differences between the experimental groups of triplicate samples were analyzed by ANOVA and Fisher's PLSD using StatView (SAS Institute Inc., Cary, NC). Values of $p < 0.05$ were considered significant.

RESULTS

Mixed Lymphocyte Reaction

We first examined the effects of EGCG treatment on allorecognition of responder T cells using one-way and two-way MLR cultures. In one-way MLR, responder T cells (C57Bl/6, H2K^b) recognized untreated stimulator cells (BALB/c, H-$2K^d$) as foreign and showed substan-

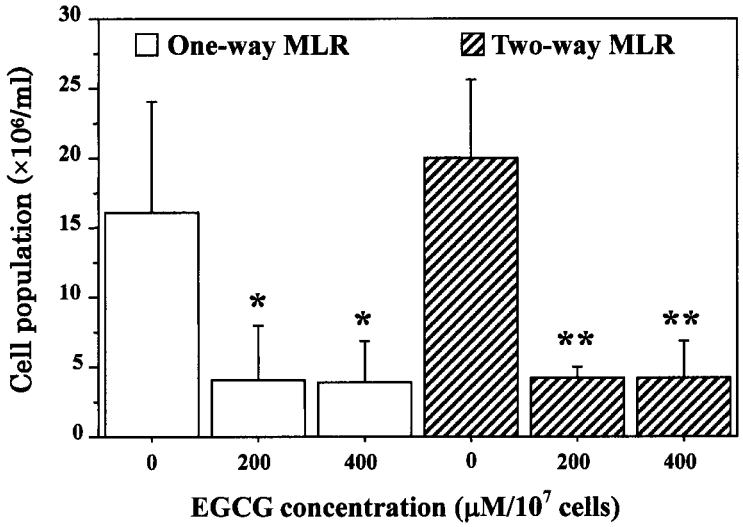

Figure 1. Effects of EGCG treatment on induction of one-way and two-way MLR. C57Bl/6 splenocytes were cocultured with EGCG-treated stimulator BALB/c splenocytes either treated (one-way MLR) or untreated with MMC (two-way MLR). Differences were significant between treated and untreated groups (*$p < 0.0001$; **$p < 0.018$).

tial proliferation (Fig. 1). The proliferation was attenuated down to 20–30% when stimulator cells were treated with 200 or 400 μM EGCG. Microscopic images of MLR cultures for these cells showed the suppressed levels of foci formation (Fig. 2). In two-way MLR, where both stimulator and responder cell populations are capable of proliferation, EGCG treatment similarly resulted in the attenuated proliferation (Fig. 1). We next examined the effects of EGCG treatment on IL-2 production in MLR cultures and found that EGCG treatment strongly reduced the production of IL-2 in one-way MLR cultures as detected by ELISA after 72 h of incubation (Fig. 3). IL-2 production was also reduced in two-way MLR cultures after EGCG treatment of BALB/c splenocytes (Fig. 3).

Detection of Cell Proliferation by Cytometry

CD71, transferrin receptor, is expressed on the cell surface of actively dividing cells and has been used as an activation marker of T-cell proliferation. To examine if the proliferation of responder T cells was attenuated by coculturing with EGCG-treated stimulator cells, cells

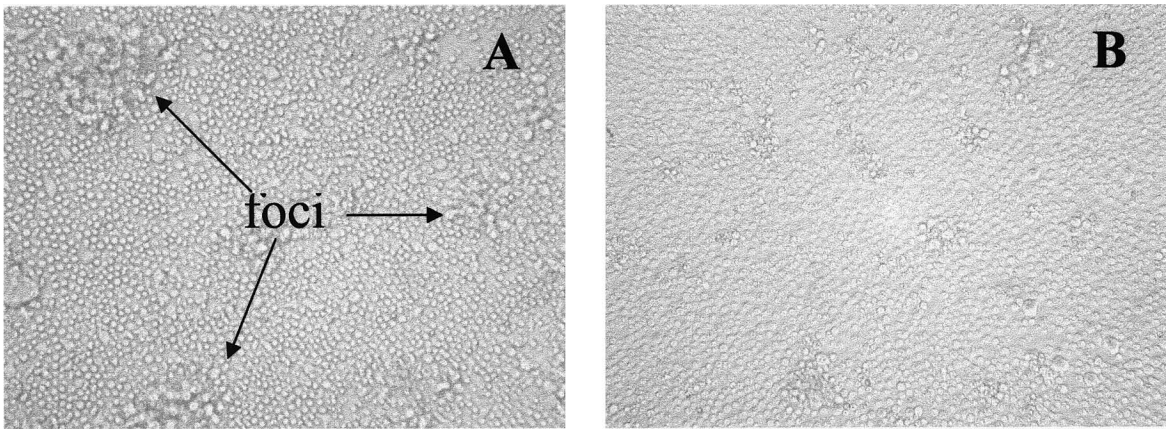

Figure 2. Foci formation of responder T cells in one-way MLR cultures. C57Bl/6 responder cells were cocultured with BALB/c simulator cells that had been either untreated (A) or treated with 400 μM EGCG (B). Microscopic images of cultured cells in a flat-bottomed 96-well plates were photographed after 72 h of incubation.

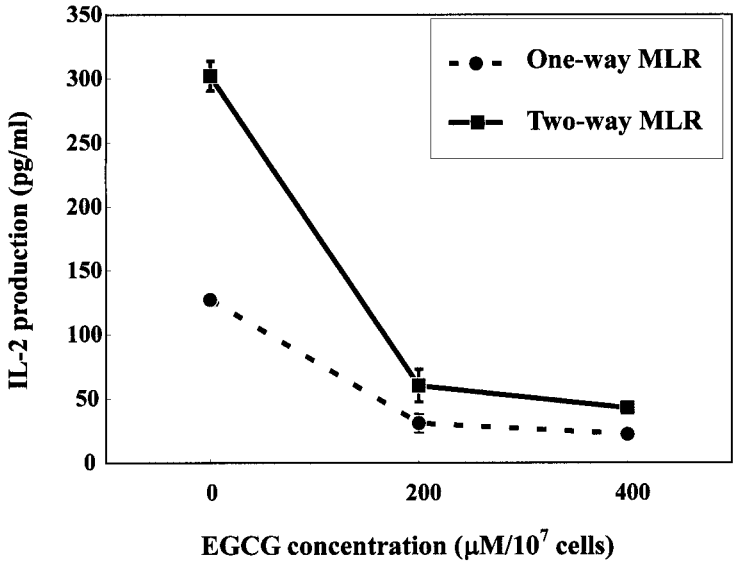

Figure 3. Inhibition of IL-2 production by EGCG treatment of stimulator cells in both one-way and two-way MLR cultures. Differences were significant between treated and untreated groups ($p < 0.0001$) for both MLRs.

from MLR culture were stained with FITC-labeled anti-mouse CD71 and PE-labeled anti-mouse Thy 1.2 antibodies, and analyzed by flow cytometry. The expression level of CD71 of EGCG-treated groups was significantly reduced down to the level of the control or the unstimulated responder cells (C57Bl/6) (Fig. 4).

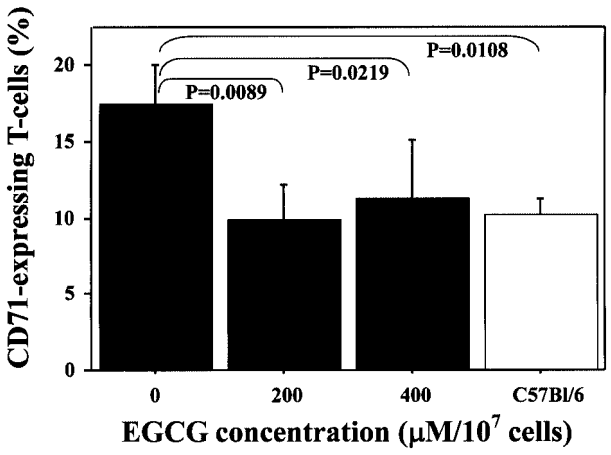

Figure 4. CD71 expression in T-cell population stimulated with EGCG-treated stimulator cells in MLR culture. Cells harvested from the MLR cultures (black columns) and from C57Bl/6 alone (white column) as a background reference were stained with FITC-anti-CD71 and PE-anti-Thy 1.2 antibodies and analyzed by FACScan.

Flow Cytometry of Cell Surface Molecules

To investigate whether the cell surface molecules of splenocytes are masked by EGCG, we performed the flow cytometric analysis of various cell surface molecules of splenocytes after EGCG treatment. Detection of most of the tested cell surface molecules was reduced by EGCG treatments, and the levels of reduction appeared to vary among different surface molecules (Table

Table 1. Detection of Cell Surface Epitopes

	EGCG Concentration (/10^7 Cells)		
Epitope	0 μM	200 μM	400 μM
TCRαβ	31.0	29.9 (3.5)	29.7 (4.2)
MHC I	96.3	92.5 (3.9)*	90.5 (6.0)*
MHC II	43.8	32.5 (25.8)*	33.2 (24.2)*
CD28	17.6	8.7 (50.6)*	9.4 (46.6)*
CD49d	42.9	19.7 (54.1)*	20.5 (52.2)*
B7.1 (CD80)	21.2	3.1 (85.4)*	6.0 (71.7)*
B7.2 (CD86)	11.6	4.2 (63.8)*	9.7 (16.4)†

Percent of epitope-positive cells measured by flow cytometry. Values in parentheses for EGCG-treated samples (200 and 400 μM/10^7 cells) represent the average percent reduction in the detection relative to the untreated control samples (0 μM/10^7 cells). Each value is an average of triplicate samples.

*$p < 0.0001$ compared with untreated controls.

†$p < 0.03$ compared with untreated controls.

1). While a moderate reduction was observed for MHC II, detection of its ligand, TCRαβ, remained unchanged (Fig. 5, Table 1). Detection of CD28, B7.1, and B7.2, which are involved in the generation of costimulatory signals for T cells, was significantly reduced by EGCG treatment (Fig. 5, Table 1). Greater reduction was also shown in CD49d, an adhesion molecule (Table 1).

Unresponsiveness of Responder T Cells Prestimulated With EGCG-Treated Stimulator Cells

To analyze whether reduced T-cell responses after EGCG treatment are reversed by secondary stimulation with fresh stimulator cells or exogenous IL-2, after one-way MLR cultures we restimulated MLR cells with

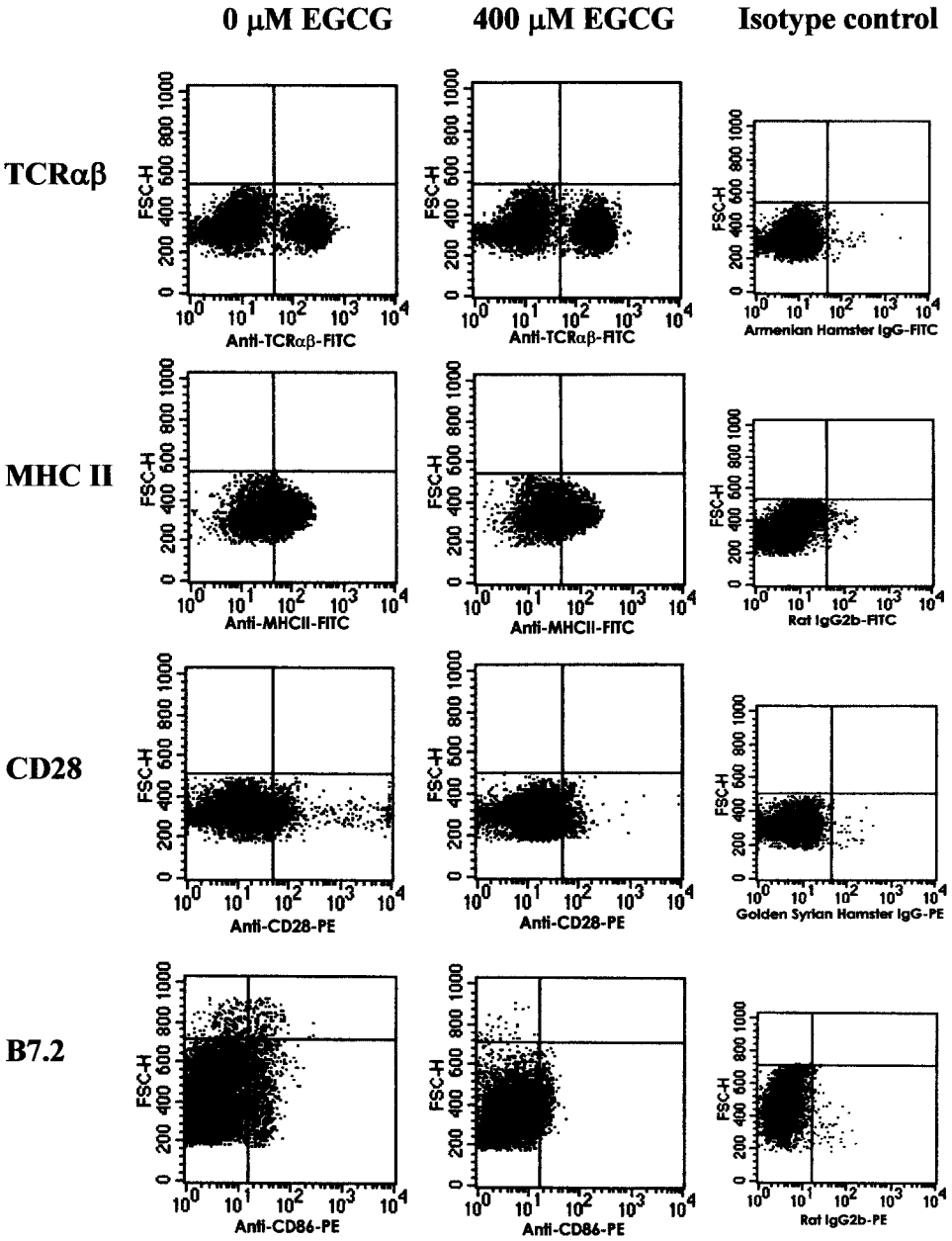

Figure 5. Flow cytometric analysis of cell surface molecules after EGCG treatment. C57Bl/6 splenocytes were either untreated or treated with EGCG (200 and 400 μM/10^7 cells), and then stained by epitope-specific monoclonal antibodies. A minimum of 10,000 counts per samples was analyzed by FACScan, and the representative results are shown.

EGCG-untreated fresh stimulator cells or IL-2 (1.7 U/ml) and analyzed the T-cell proliferation in secondary cultures. There was significant suppression of T-cell proliferation down to or below that of the unstimulated control cultures (Fig. 6).

Detection of Apoptosis by Cytometry

To examine the proportions of apoptotic cells in responder cells (C57Bl/6) and EGCG-treated stimulator cells (BALB/c), cells from MLR cultures after 24 h were analyzed by Annexin V assay. For the responder cells that were cocultured with the stimulator cells treated with EGCG, there was no substantial change in the proportion of apoptotic cells due to the EGCG treatment (0 μM: 81.8 ± 2.1%; 200 μM: 77.9 ± 1.1%; 400 μM: 78.7 ± 1.7%, based on one-way ANOVA of triplicate samples, $F = 4.43$, $p > 0.05$) (Fig. 7). In contrast, for the stimulator cells, the proportion of apoptotic cells significantly decreased with the EGCG treatment (Fig. 7).

DISCUSSION

Our present study demonstrated that EGCG can prevent allostimulation in a murine in vitro allograft context. EGCG treatment of murine splenocytes decreased the cell signaling for optimal allostimulation and attenuated the activation and proliferation of T cells in the MLR cultures.

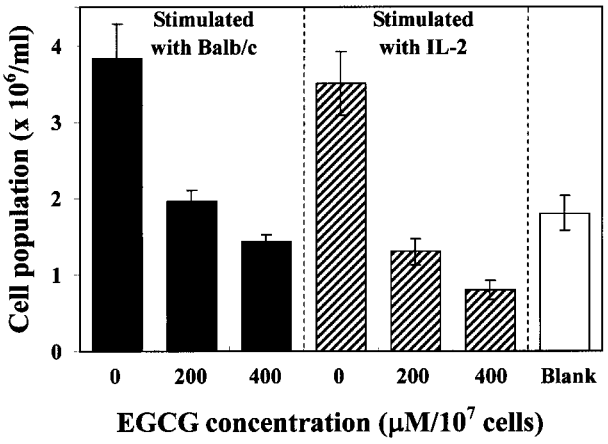

Figure 6. Unresponsiveness of responder T cells that have been prestimulated with EGCG-treated stimulator cells. After coculturing C57Bl/6 splenocytes with EGCG-treated BALB/c splenocytes for 48 h, MLR wells were restimulated with either fresh untreated BALB/c splenocytes (black columns) or exogenous IL-2 (hatched columns), and incubated for an additional 48 h. C57Bl/6 without any stimulation is shown as a background (blank column). Differences were significant between untreated and treated groups (200 or 400 μM/10^7 cells) for cell-stimulated cultures ($p < 0.0001$) and IL-2-stimulated cultures ($p < 0.0001$).

Tissue or organ transplantation is increasingly applied for the treatment of many medical problems, but its major drawbacks are transplant rejection and GVHD. One of the ordinary therapeutic methods to prevent these problems is the application of pharmacological agents that suppress T-cell activation (18). Although these chemical agents mitigate the problems by inhibiting T-cell signaling and activation, they often cause significant side effects on various tissues such as renal, hepatic, gastrointestinal, and mucosal systems (3). To avoid these side effects, more amenable methods have been developed (10,11,14). One of the promising methods is immunocamouflage where the donor tissues bearing cell surface antigenic determinants are coated with nonimmunogenic agents such as polyethylene glycol (PEG) (14). PEG can block immunogenic recognition by the host immune system and decrease graft rejection and GVHD (4,13). In the present study, we hypothesized that a tea polyphenol, EGCG, also blocks antigenic determinants and attenuates the allostimulation and subsequent activation of host T cells.

Our results indicate that responder T-cell proliferation in the MLR culture was attenuated when the stimulator cells were treated with EGCG (Fig. 1). The EGCG treatment of the stimulator cells representing the transplant led to the attenuation of T-cell proliferation of the responder cells (Fig. 4) representing the host, which is usually targeted by immunosuppressive drugs. Because the stimulator cells in one-way MLR culture had been arrested of proliferation by mitomycin C, the decrease of T-cell proliferation was largely due to the influence of EGCG on the stimulator cells and/or their stimulatory activities. The choice of C57Bl/6 as the responder rather than the stimulator in the one-way MLR in these experiments was to avoid overestimating the attenuating potential of EGCG treatment because C57Bl/6 T cells are classified as Th-1 type and are generally considered to have stronger and more sustained response toward cellular antigens than BALB/c T cells, which belong to Th-2 type.

It must be noted that the decrease of T-cell proliferation was not due to the direct toxicity of EGCG on the stimulator cells. In fact, EGCG-treated stimulator cells in MLR cultures had reduced levels of apoptosis than untreated controls (Fig. 7). For the same reason, the production of IL-2 in the one-way MLR cultures decreased supposedly due to the reduced stimulation of T cells (Fig. 3) by the EGCG-treated stimulator cells but not loss of viability of the latter. IL-2 production was also reduced in two-way MLR cultures (Fig. 3). In two-way MLR cultures, we cannot simply determine which cell type (C57B/6 or BALB/c) is actually contributing to IL-2 production, and this reduction seemed assigned to the inactivation of both responder and stimulator T cells,

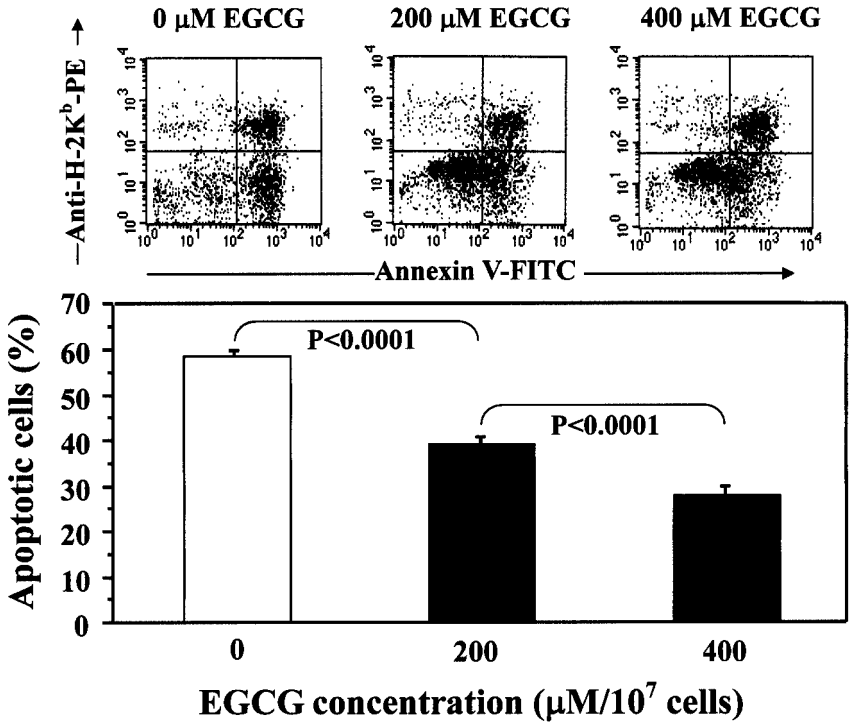

Figure 7. Analysis of apoptosis in responder and EGCG-treated stimulator cells in the MLR culture. C57Bl/6 splenocytes were cocultured with EGCG-treated BALB/c splenocytes for 24 h, and cultured cells were stained with PE-conjugated anti-mouse H-2K^b and FITC-conjugated Annexin V. The upper figures show three representative scatter grams (lower left quadrant: nonapoptotic stimulator; lower right quandrant: apoptotic stimulator; upper left quadrant: nonapoptotic responder; upper right quadrant: apoptotic responder cell populations). The lower graph shows the proportions of stimulator cell populations treated with 0, 200, or 400 μM EGCG before MLR culture with responder cells.

because activation of stimulator (BALB/c) T cells was also attenuated by the treatment with EGCG (our unpublished results).

The T cells stimulated with EGCG-treated stimulators were not only attenuated of proliferation but rendered somehow unresponsive toward allostimulation (i.e., they were in an anergic state). In fact, the proliferation of the responder cells did not recover upon secondary stimulation with fresh stimulator cells or even exogenous IL-2 (Fig. 6). The lack of responsiveness to the secondary stimulation with cells is not necessarily due to the mortality of responder cells, as indicated by the result of apoptosis detection (Fig. 7). Although T-cell apoptosis is known to be required in the induction of peripheral transplantation tolerance (20), the level of responder cell apoptosis occurring in our experimental system suggests that the induction of anergy due to EGCG treatment of stimulator cells is involved in the unresponsiveness of responder T cells (Fig. 7) Furthermore, the lack of responsiveness to the secondary stimulation with exogenous IL-2, in turn, suggests that the stimulation of CD8$^+$ T cells may be prevented by the stimulation with EGCG-treated stimulators. These results provide further evidence to support that EGCG treatment prevents acute graft rejection (7) and present a question as to what mechanisms are involved in the influence of EGCG on the stimulator cells and their activities.

In light of the present hypothesis, the simplest interpretation is that stimulatory activities were abrogated by blocking cell surface molecules with EGCG, resulting in weak or incomplete stimulation of alloreactive T cells. Indeed, staining intensity of cell surface molecules involved in allorecognition, costimulation, and cell-to-cell adhesion were reduced by the EGCG treatment (Table 1). Weakening or blockage of interactions of any of these molecules and their ligands could lead to incomplete activation of T cells (4).

Particular attention is paid to the different effects exhibited among these cell surface molecules. Normally,

T cell is stimulated at an allorecognition site that constitutes a main recognition domain of T-cell receptor (TCR) that binds to a complex formed by a foreign peptide and a major histocompatibility complex class I or II (MHC I or II) molecule. As the results of surface marker analysis indicate, on one hand, EGCG treatment moderately reduced the detection of MHC I and II molecules but not that of TCRαβ (Fig. 5, Table 1). On the other hand, EGCG treatment strongly reduced the detection of costimulatory molecules B7.1 (CD80) and B7.2 (CD86) as well as their ligand, CD28 (Fig. 5, Table 1), indicating potential abrogation of pivotal costimulatory signals (6,8,15–17) that may lead to the induction of anergic state in T cells. The detection of adhesion molecule, CD49d, was also strongly reduced, suggesting weakening of cell-to-cell adhesion prerequisite to allostimulation (Table 1).

Besides the anergy induction, other modes of action of EGCG can be suggested with respect to its possible influence on antigen-presenting cells. The EGCG molecules, with their amphipathic property depending on treatment conditions, are considered to penetrate the cell membrane and interfere with specific biochemical pathways underlying cell activation processes. For example, EGCG is known to downregulate the activity of NF-κB, which is involved in the activation of macrophages and dendritic cells (1,9). In addition, there is a large number of cytokines that are involved in the activation of antigen-presenting cells, and EGCG may influence the activity or production of these cytokines. EGCG may not only influence these indirect immunosuppressions but also the direct suppression by means of regulatory T cells.

The EGCG treatment presented in this study can provide a novel and useful method to prevent allorejection of foreign tissues, because donor tissues are simply to be immersed in culture media containing EGCG before being transplanted to a recipient body system. In terms of its potential clinical application, this plant-derived agent is expected to require less preclinical toxicological tests and lower manufacturing costs than synthetic chemicals. Regarding dose–effect comparison, EGCG dosage used in the present study was somewhat higher than that of methylated PEG, but the overall level of the attenuation effect was in a similar range to that reported for PEG-camouflaged cells (4). Although further investigation is necessary with respect to the mechanisms behind the immunosuppressive actions, the treatment of donor tissues with EGCG may have a potential value for the prevention of allorejection and can be applicable in other transplantation situations.

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