

In vitro study of immune tolerance induced by CTLA4-Ig in bone transplantation: The effect on cell proliferation stimulated by lymphocytes and bone supernatant

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ABSTRACT

To clarify the effect of CTLA4-Ig on immune rejection of bone grafts, we observed the effect of CTLA4-Ig on lymphocyte proliferation of BALB/C mice stimulated by lymphocytes and bone supernatant of C57BL/6 mice. The splenic lymphocytes and bone supernatant of C57BL/6 mice, as the stimulator cells and stimulator antigens, were cultured in vitro with the splenic lymphocyte of BALB/C mice. At the same time, CTLA4-Ig at a dose of 5, 10 or 20 µg/ml and L6 (as control) at 20 µg/ml were added. Six days later, the incorporation of ³H-TdR was determined. Results indicated that CTLA4-Ig at a dose of 5, 10 or 20 µg/ml significantly inhibited the cell proliferation stimulated by lymphocytes and bone supernatant of C57BL/6 mice. The effect was non-cytotoxic. L6 showed no significant inhibition of cell proliferation. CTLA4-Ig can efficiently block the proliferation of alloresponsive T cell stimulated by lymphocytes and bone supernatant of C57BL/6 mice. This study provides a

basis for further study of CTLA4-induced immune tolerance of bone grafts.

Key words: CTLA4-Ig, immune tolerance, lymphocyte, bone supernatants, mouse

INTRODUCTION

At present, the main problem of organ transplantation is immune rejection. The transplantation antigens evoke an immune response of the recipient by two signals: one, antigen signal, the other, costimulatory signal, i.e. CD28/CTLA4 with B7 ligands. If there is only the engagement of the antigen signal, with the blockade of the costimulatory signal, T-cell would have a response of anergy or apoptosis. CTLA4-Ig, as a soluble form of CTLA4, can induce tolerance in some animal transplant models, but knowledge of the effect of CTLA4-Ig on bone grafts is still lacking. This study was designed to investigate the efficacy of CTLA4-Ig on cell proliferation stimulated by lymphocytes and bone supernatant, with the aim of exploring the immunosuppressive capacity of CTLA4-Ig.

MATERIALS AND METHODS

1. Animals and Reagents

One Inbred male C57BL/6 and 5 BALB/C mice were used at 10–18 weeks of age. The animals weighed 20–22 g.

2. Isolation of Splenic Lymphocytes

The spleen was obtained after the mouse was killed under sterilization. Then it was minced, filtered, separated by a Ficoll fluid ($d=1.088$), and the cells were counted. The cell concentration was adjusted to 2×10^6 /ml of stimulator cells and 4×10^6 /ml of responder cells. The stimulator cells were irradiated by 3000 rad ^{60}Co to eliminate the ability of mitosis.

3. Bone Supernatant

The methods of Sun Lei⁴ were adopted. Both leg bones of one C57BL/6 mouse were obtained and minced after the soft tissue and bone marrow were stripped. Then it was ground into the homogenate in a homogenizer, and 12 ml RPMI1640 media were added. After mixing, the mixture was kept still for 1 hour. The supernatant was obtained.

4. Mixed Leukocyte Culture (MLC)

One-way MLC was performed, where equal volumes (100 μl) of responder cells (4×10^5) from BALB/C mice and stimulator cells (2×10^5) from C57BL/6 mouse were cocultured (triplicate) in 96-well flat-bottom microtiter plate (Costar, Cambridge MA) at 37° in 5% CO₂. Five pairs of mice and six groups of each pair were arranged as follows:

- responder cells + medium only, Abbr. B+1640
- responder cells + stimulator cells, Abbr. B+C
- responder cells + stimulator cells+L6 (20 $\mu\text{g}/\text{ml}$), Abbr. B+C+L6 (20)
- responder cells + stimulator cells+CTLA4-Ig (5 $\mu\text{g}/\text{ml}$), Abbr. B+C+CTLA4-Ig (5)
- responder cells + stimulator cells+CTLA4-Ig (10 $\mu\text{g}/\text{ml}$), Abbr. B+C+CTLA4-Ig (10)
- responder cells + stimulator cells+CTLA4-Ig (20 $\mu\text{g}/\text{ml}$), Abbr. B+C+CTLA4-Ig (20)

After 5 days of incubation in RPMI1640 medium with 5 mM HEPES, $5 \times 10^{-5}\text{M}$ 2-ME, 100 $\mu\text{g}/\text{ml}$ penicillin, 100 $\mu\text{g}/\text{ml}$ streptomycin, and 10% FCS, the cells were pulsed with 1 $\mu\text{Ci}/\text{well}$ of $^3\text{HTdR}$ for the last 16h of culture. The cells were then harvested with a cell harvester, and their proliferative ability was assessed by measuring $^3\text{HTdR}$ incorporation rate. Experiments were performed in quadruplicate. A student t test was used for statistical analysis. The results were expressed as $\bar{x} \pm s$ of 5 BALB/C mice. If stimulator cells were replaced by bone supernatant

(BS), the experimental methods would remain the same as above.

- In addition, the group of B+C+CTLA4-Ig(20) was designed to determine the cytotoxicity of CTLA4-Ig with trypan blue.

RESULTS

1. The effect of CTLA4-Ig on cell proliferation stimulated by allo-lymphocyte

As shown in Table 1, splenic cell proliferation of BALB/C mice was significantly stimulated by the splenic cells of a C57BL/6 mouse ($P < 0.01$). L6 had no effect on lymphocytes proliferation ($P > 0.05$), but CTLA4-Ig significantly inhibited lymphocyte proliferation, whether its concentration was 5, 10 or 20 $\mu\text{g}/\text{ml}$ ($P < 0.001$). By comparison, CTLA4-Ig at a dose of 20 $\mu\text{g}/\text{ml}$ had a mildly remarkable inhibitory effect.

2. The effect of CTLA4-Ig on cell proliferation stimulated by allogeneic bone supernatant

As shown in Table 2, bone supernatant of C57BL/6 mice had no significant effect on stimulation of lymphocytes of BALB/C mice ($P > 0.05$). L6 had no effect, but CTLA4-Ig significantly inhibited the lymphocytes proliferation of BALB/C mice ($P < 0.001$). There was no difference between different doses of CTLA4-Ig ($P > 0.05$).

- As shown by trypan blue staining, even if CTLA4-Ig was added to the medium at a dose of 20 $\mu\text{g}/\text{ml}$, more than 95% of the cells still survived. This indicated that CTLA4-Ig only inhibits cell proliferation, but has no cytotoxic effect.

Table 1
The effect of CTLA4-Ig on BALB/C lymphocyte proliferation stimulated by C57BL/6 lymphocyte

No.	n	Groups	Responder cells	$^3\text{H-TdR}$ Incorporation rate
1	5	B+1640	4×10^5	7639 \pm 2056
2	5	B+C	4×10^5	13807 \pm 3021
3	5	B+C+L6	4×10^5	14061 \pm 2960
4	5	B+C+CTLA4-Ig(5)	4×10^5	1427 \pm 400
5	5	B+C+CTLA4-Ig(10)	4×10^5	1263 \pm 342
6	5	B+C+CTLA4-Ig(20)	4×10^5	1092 \pm 341

Note: 'n' expresses the numbers of animals as the responder cells.

Table 2
The effect of CTLA4-Ig on BALB/C lymphocyte proliferation stimulated by C57BL/6 bone supernatant

No.	n	Groups	Responder cells	³ H-TdR Incorporation rate
A	5	B+BS	4X10 ⁵	6472±1775
B	5	B+BS+L6	4X10 ⁵	6800±1968
C	5	B+BS+CTLA4-Ig(5)	4X10 ⁵	702±153
D	5	B+BS+CTLA4-Ig(10)	4X10 ⁵	786±165
E	5	B+BS+CTLA4-Ig(20)	4X10 ⁵	924±153

Note: 'n' expresses the numbers of animals as the responder cells.

Table 3
The results of student t test

Groups	P	Groups	P
2:1	< 0.01	A:1	> 0.05
3:2	> 0.05	B:A	> 0.05
4:2	< 0.001	E:A	< 0.001
4:3	< 0.001	E:B	< 0.001
4:6	> 0.05	E:C	> 0.05

DISCUSSION

Although bone transplants have been extensively used in the field of orthopaedics, oral surgery and plastic surgery, there are some problems that still remain unresolved, such as immune rejection, infection, fracture and bone sources. The methods to preserve bone grafts such as irradiation, freezing and lyophilization, may reduce the immunogenicity of the bone but cause the loss of its inductivity, leading to delayed healing. The immunosuppressive drugs can improve the incorporation of bone grafts, but the systemic immune system is also inhibited.² It is perplexing how to resolve the contradiction of decreasing the immunogenicity without disturbing normal immune function. Recently, it has been shown that CTLA4-Ig induces donor-specific tolerance in rodent organ transplantation, namely the immune

tolerance only to transplantation antigen. This can avoid the deficit of immunosuppressive drugs and retain the capacity of osteoinduction.

Since Brunet¹ discovered that CTLA4 was a cell-surface antigen, more and more studies have been made to investigate its functional mechanisms. It is accepted that CTLA4 is an inhibitory signal for CD28-B7 costimulation pathway. CTLA4 is very important in regulating the homeostasis of lymphocyte proliferation. CTLA4-Ig, as a soluble form of CTLA4, has been used to treat autoimmune disorders and transplantation rejection.

So far, there is no report on applying CTLA4 to bone graft. In this study, we observed the effect of CTLA4-Ig on cell culture in vitro. The results show that there is a significant stimulating effect on proliferation in MLC of different strains of mice (C57BL/6 and BALB/C) (P < 0.01). When CTLA4-Ig was added, cell proliferation stimulated by lymphocyte or bone supernatant is significantly inhibited (P < 0.001). Moreover, the higher the concentration, the more obvious the inhibition is. The dose-dependent relation has been shown by experimental study of Tepper.⁵ Staining with trypan blue proved that the inhibition is non-cytotoxic. L6, which is an immunoglobulin, has no effect on cell proliferation. This indicates that the effect of CTLA4-Ig is CTLA4 but not Ig. What is more important, we also observed the significantly inhibitory effect of CTLA4-Ig on cell proliferation stimulated by bone supernatant. This verifies that CTLA4-Ig can block the cellular immune response stimulated by bone antigens. The study provides a basis for further study of immune tolerance of bone grafts. The advances in research of CTLA4-Ig on organ transplantation would be expected to resolve the problem of immune rejection in human organ transplantation.

CONCLUSION

Through this study it was found that the cell proliferation ability, among allogeneic mice, can be inhibited by CTLA4-Ig whether it was lymphocyte or bone supernatant. So we can predict that CTLA4-Ig may play a similar role in vivo experimental study. As soon as the immune tolerance in bone allograft can be successfully induced, we would be able to improve the success rate of bone grafts through using fresh bone allograft.

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*This project was supported by the State Natural Sciences Fund (Approval No. 39670725). The mice came from the Experimental Animal Center of Shanghai Second Medical University. The CTLA4-Ig and L6 were graciously provided by Bristol-Myers Squibb Pharmaceutical Research Institute, Seattle, USA.