

## Expression of Amino Acid Transporter LAT1 and 4F2hc in the Healing Process after the Implantation of a Tooth Ash and Plaster of Paris Mixture

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**Abstract.** *Background:* In order to elucidate the expression pattern of L-type amino acid transporter 1 (LAT1) in the bone formation process, the expressions of LAT1 and its subunit 4F2 heavy chain (4F2hc) were investigated in the healing process after the implantation of a tooth ash-plaster of Paris mixture in rats with calvarial osseous defect. *Materials and Methods:* Circular calvarial defects (8 mm in diameter) were made midparietally. The rats were divided into 2 groups, 1 control group and 1 experimental group. In the control group, the defect was only covered with a soft tissue flap (control group); in the experimental group, it was filled with a mixture of tooth ash and plaster of Paris (2:1 by weight; mixture group). The rats were sacrificed at 1, 2, 4 and 8 weeks after operation and RT-PCR and immunohistochemical analyses were performed. *Results:* In the RT-PCR analysis, the expressions of the LAT1 and 4F2hc mRNAs were slightly stronger in the experimental group than in the control group. In the immunohistochemical analysis, at 1 week after operation, the LAT1 protein and its subunit 4F2hc protein were mainly expressed in the osteoblasts, osteocytes and interstitial tissues of the area around the defect and the inner part of newly forming bone in both groups. The expressions of LAT1 and 4F2hc proteins were decreased at 2 and 4 weeks after operation. The LAT1 and 4F2hc proteins were scarcely expressed at 8 weeks after operation in both groups. The expressions of LAT1 and 4F2hc proteins were slightly stronger in the mixture group than in the control group. *Conclusion:* These results suggest that the LAT1 and its subunit 4F2hc are highly expressed in the early stage of new bone

formation and may have an important role in providing cells with neutral amino acids, including several essential amino acids, at that stage.

Autogenous bone grafts have been widely used in oral and maxillofacial surgery. They offer a range of advantages including early revascularization, resistance to infection and evidence of immune activation. However, autogenous bone grafts also have several disadvantages: limitation of graft volume available, donor site morbidity and prolongation of the operation. Thus, a wide variety of alternative materials has been used including allografts, xenografts and alloplastic implants (1).

Particulate dentine (tooth ash) is derived from sound teeth and is composed mainly of hydroxyapatite. It has been used as an alternative material for implant with bioresorbable properties. However, the use of particulate dentine presents a problem in retention of the graft material (2, 3). Calcium sulphate ( $\text{CaSO}_4 \cdot 1/2\text{H}_2\text{O}$ ), commonly known as plaster of Paris, has also been used as an alloplastic implant in maxillofacial osseous defects. It seemed to be promising because of its long history of safe use and its characteristically complete resorption followed by new bone formation (4-7). It has been proposed as an effective binding and stabilizing agent for particulate materials (8). A mixture of tooth ash and plaster of Paris has been studied as a new biomaterial; its biocompatibility and the possibility of repairing bony defects with this mixture has been explored (3, 9). We have used this mixture clinically in 10 patients and obtained successful results with few complications (9). High density new bone formation is essential for the osteoinduction and osteogenesis induced by graft materials, and high protein synthesis may be essential for this.

Amino acids are indispensable for protein synthesis and consequently essential for cell growth and proliferation in both normal and transformed cells (10, 11). Amino acid transport across the plasma membrane is mediated *via* amino

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acid transporters located on it (10, 11). The continuous growth and proliferation of cells requires continuous protein synthesis. It is known that the supply of amino acids for continuous protein synthesis in these cells is mediated by the overexpression of amino acid transporters (12-14).

Among the amino acid transport systems, amino acid transport system L, which is an  $\text{Na}^+$ -independent neutral amino acid transport system, is a major route for providing living cells, including tumor cells, with neutral amino acids including several essential amino acids (10, 11). Recently, the system L-type amino acid transporter 1 (LAT1) was isolated (12, 13). It was predicted to be 12-membrane-spanning proteins that mediate  $\text{Na}^+$ -independent amino acid exchange (12, 13). It requires an additional single-membrane-spanning protein, a heavy chain of 4F2 antigen (4F2hc), for its functional expression in the plasma membrane (12, 13, 15-18). The LAT1 is highly expressed in growing and proliferating cells, such as malignant tumor cells, presumably to support their continuous growth and proliferation (12-14).

It is reported that the L-type amino acid transport system may be important in a living organism, because it is a major route for providing living cells with neutral amino acids including several essential amino acids, which cells are unable to synthesize (10, 11). Thus, the system L may have an essential role in the bone regeneration process. However, the expression and functional characterization of amino acid transporters, including the system L, in supplying nutrition to cells in the bone regeneration process are not known.

In the present study, in order to elucidate the role of LAT1 in the bone regeneration process, the expression pattern of LAT1 and its subunit 4F2hc in the healing process after the implantation of a tooth ash-plaster of Paris mixture in rats with calvarial osseous defect was investigated. The LAT1 and its subunit 4F2hc were highly expressed in the early stage of new bone formation and may have an important role in providing cells with neutral amino acids, including several essential amino acids, at that stage.

## Materials and Methods

**Materials.** Tooth ash was prepared from healthy teeth, extracted from humans, by washing the teeth in saline solution, reducing the teeth to ash in a furnace at 1,200°C, and grinding the ash into a powder using a mesh tray (sieve no. 100; 0.149 mm) (19). This tooth ash was mixed with plaster of Paris (Dental Plaster; Mungyo Industrial Co., Gimhae, Korea) at a weight ratio of 2:1. All materials were sterilized with ethylene oxide before implantation and physiological saline solution was used for mixing the implant.

Affinity-purified anti-LAT1 and anti-4F2hc polyclonal antibodies were kindly provided by Kumamoto Immunochemical Laboratory, Transgenic Inc. (Kumamoto, Japan). Other chemicals were purchased from Sigma (St. Louis, MO, USA).

**Animals.** This study was approved by the Animal Research Committee of Chosun University. Sprague-Dawley rats weighing 200-250 g were selected for the study. The rats were housed under controlled conditions (22°C, 12-h light/dark cycle) and fed with standard laboratory chow and tap water *ad libitum*.

An 8-mm-diameter calvarial critical size defect was created in each rat (20). A critical size defect may be defined as the smallest size of an intraosseous wound, in a particular bone and species of animal, that will not heal during the lifetime of the animal (21). A defect in the skull measuring 8 mm in diameter requires 0.25 g of tooth ash.

The rats were divided into an experimental group that was given the implant (the mixture of tooth ash and plaster of Paris) and a control group that that was not. Each group was further subdivided into the groups that were sacrificed at 1, 2, 4 and 8 weeks after surgery. The rats were divided into a total of 8 groups, with each group containing 8 rats.

**Defect creation and implantation.** General anesthesia was induced in rats by intramuscular co-injection of ketamine HCl (10 mg/kg; Yuhan, Seoul, Korea) and xylazine HCl (10 mg/kg; Bayerkorea, Gyeonggi, Korea). The head was shaved and sterilized according to conventional methods, and 2% lidocaine HCl containing 1:100,000 epinephrine was injected for hemostatic purposes. An incision was made along the middle portion of the skull to expose the brain. With the sutural site avoided at the middle, a hole was made in the skull of 8 mm diameter by removing the entire layer of the skull using a round bur. The previously prepared mixture of tooth ash and plaster of Paris was used to close the defect. The skull was then sutured back. The control rats were treated as the experimental rats by making a bone defect, but this was sutured back without implantation. Intramuscular injection of 2 mg/kg gentamicin (Daesung Microbiological Labs Co., Gyeonggi, Korea) was performed to prevent infection after surgery. The rats were sacrificed at 1, 2, 4 and 8 weeks after surgery.

**Histological analysis.** After each rat was sacrificed *via* excess ether inhalation, a bone sample was obtained from around the implant site, fixed in 10% neutral formalin for 72 h and decalcified in nitric acid for 4 h. This bone sample was cut into 3-mm-thick sections which were washed in running water. Each bone tissue was treated using an autoproccessing machine (Hypercenter XP, Shandon, UK). After being embedded in paraffin, each section was cut into 5- $\mu\text{m}$  slices which were then stained in hematoxylin-eosin and Goldner's trichrome and observed under an optical microscope.

**Reverse transcription-polymerase chain reaction (RT-PCR) analysis.** The total RNAs were prepared from the tissues from the implant site using RNA preparation kits (Isogen, Nippon-gene, Japan), in accordance with the manufacturer's instructions. For RT-PCR analysis, the first-strand cDNAs were prepared from the total RNAs using the Superscript first-strand synthesis system for RT-PCR (Life Technologies Inc., CA, USA) with an oligo dT primer and were used as a template for PCR amplification. PCR amplification was performed using Taq Polymerase Aamplitaq Gold (Roche Molecular Systems, Inc., Germany) according to the following protocol: 94°C for 12 min, followed by 25 cycles at 94°C for 30 sec, 60°C for 30 sec and 72°C for 40 sec and a final extension step of 72°C for 30 min. The primers, 5'-CAATGGTGTGGCCATCATAG-3' (162-181 bp of

coding region) and 5'-GATGCATCCCCTTGTCTAT-3' (670-689 bp of coding region), were used for PCR amplification of rat LAT1. The primers, 5'-TCACAGGCTTATCCAAGGAG-3' (161-180 bp of coding region) and 5'-TACAATGTCAGCCTGAGGAG-3' (641-660 bp of coding region), were used for PCR amplification of rat 4F2hc. The PCR products were electrophoresed on a 1.5% agarose gel and visualized with ethidium bromide.

**Immunohistochemistry.** The previously fixed tissues were embedded in paraffin. Sections of 5- $\mu$ m were made, mounted on poly-L-lysine coated glass slides and dried overnight at room temperature. After the sections were deparaffinized in xylene and rehydrated through graded ethanol, they were immersed in 3% hydrogen peroxide in methanol (v/v) for 15 min in order to quench endogenous peroxidase activity. They were then washed in TRIS and incubated with normal 1% BSA (bovine serum albumin in TRIS) for 1 h to reduce nonspecific binding of primary antibody. After being washed in TRIS, they were incubated with anti-LAT1 (1:100 dilution) or anti-4F2hc (1:100 dilution) affinity-purified primary antibody. Thereafter, they were treated with secondary antibodies for 30 min at room temperature. To detect immunoreactivity, the sections were treated with diaminobenzidine (0.8 mM) and counterstained with hematoxylin. The protein expression of LAT1 and 4F2hc was defined immunohistochemically as: + + +, strong expression; + +, moderate expression; +, weak expression; +/-, doubtful expression; -, no expression.

## Results

### *Histological observations*

*(i) Control group:* first week-findings: The connective tissue was characterized by acute inflammatory reactions with the formation of granulation tissue, characteristic of immature fibroblasts, vascularization and edema. In bone near to the bone defect, there was slight new bone formation and osteoblastic activity.

Second week-findings: There was a reduction in inflammation and edema; active cellular proliferation of fibroblasts was observed. In bone near to the bone defect, new bone columellization was active. Osteoblast activity was increased around the bone trabecula.

Fourth week-findings: Compared to the second week, a significant decrease in inflammation and edema was observed in connective tissue; significant increased proliferation of fibroblasts and deposition of collagen was observed. In bone near to the bone defect, there was active new bone columellization and an increase in the amount of new bone. A faint reversal line was observed.

Eighth week-findings: The bone defect was filled with compact fibrous connective tissue which had fibroblasts and dense collagen. In bone near to the bone defect, the amount of new bone was greater and a distinct reversal line was seen compared to the fourth week.

*(ii) Mixture group:* first week-findings: There was heavy chronic inflammatory cell infiltration and foreign-body giant cells. The connective tissue showed weak edema, proliferation of

immature fusiform fibroblasts and vascularization around the implant materials. In the periphery of the pre-existing bone around the implant materials, there was a small quantity of newly formed bone, but it was separate from the implant materials.

Second week-findings: Around the implant materials, connective tissue edema was reduced, but vascularization and fibroblastic proliferation was more active compared to the first week. It was observed that there was active proliferation of new bone trabecula in the bone defect; osteoblastic activities were increased in the periphery; in some cases, a small quantity of thin osteoid tissue was observed around the implant materials.

Fourth week-findings: The inflammatory reaction was reduced in the connective tissue around the implant materials. The connective tissue revealed vascularization and active proliferation of fibroblasts. The bone defect was completely covered with new bone trabecula without the connective tissue entrapment, which was fused with the pre-existing bone. In the newly-formed bone, there were distinct reversal lines and active osteoblastic rimming in the periphery.

Eighth week-findings: Compared to the fourth week, inflammatory reaction in the connective tissue had decreased and fibroblastic proliferation had increased. In the bone defect area, it was difficult to distinguish the pre-existing bone for it was covered with new bone trabecula around the implant materials. Also, it could be distinctive only with reversal lines and the implant in internal new bone. Moreover, it was observed that new bone trabecula had proliferated up to the isolated implant materials.

**RT-PCR analysis.** In the RT-PCR analysis, the PCR products for LAT1 and its associated 4F2hc were detected at 1, 2, 4 and 8 weeks in the control and experimental graft groups (Figure 1). The signals for the products of the LAT1 and 4F2hc were slightly stronger in the experimental group than in the control group. In both control and experimental groups, the signals for the products of the LAT1 and 4F2hc decreased after implantation in a time-dependent manner (Figure 1).

**LAT1 and 4F2hc expression.** The immunohistochemical results of LAT1 and 4F2hc protein expression are shown in Table I and Table II, respectively.

*(i) Control group:* In the first week, LAT1 protein was moderately expressed around the newly formed bone in the bone defect, and in osteoblasts, osteocytes and interstitial tissue in the pre-existing bone (Figure 2). As time progressed, the expression of LAT1 decreased. In the eighth week, expression was low. 4F2hc protein was weakly expressed around the newly-formed bone in the bone defect, and in osteoblasts, osteocytes, and interstitial tissue in the pre-existing bone (Figure 3). It was very weakly expressed at 2, 4, and 8 weeks.

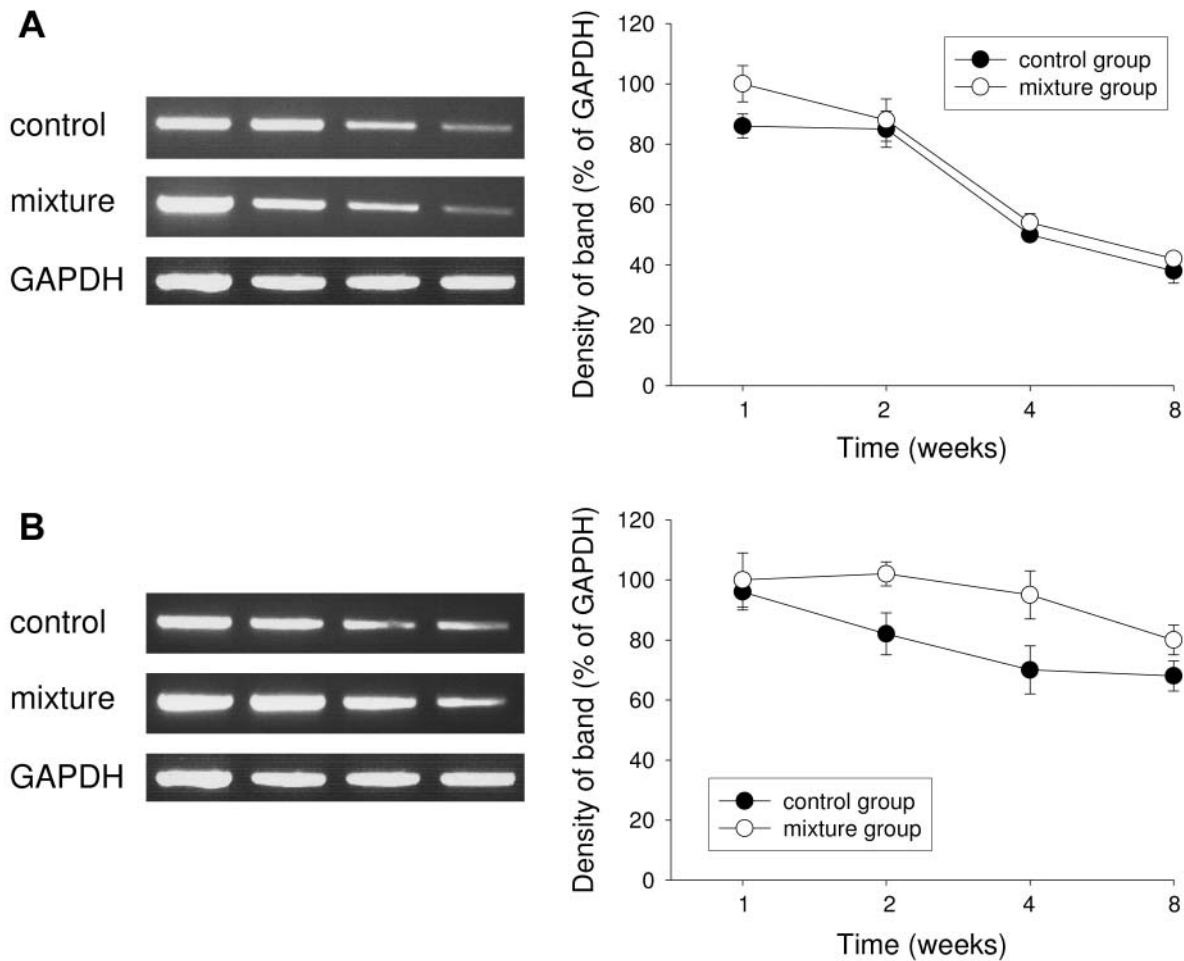


Figure 1. Detection of *LAT1* and *4F2hc* mRNAs using RT-PCR. The first strand cDNAs prepared from the control group and the tooth ash-plaster of Paris mixture group total RNAs were used as a template for PCR amplification. The PCR products were electrophoresed on a 1.5% agarose gel and visualized with ethidium bromide. A. The left panel shows the *LAT1*-specific PCR product (527 bp) obtained from the control and mixture groups. The right panel shows the percentage of *LAT1* mRNA expression as calculated as a ratio of the *GAPDH* band. B. The left panel shows the *4F2hc*-specific PCR product (499 bp) obtained from the control and mixture groups. The right panel shows the percentage of *4F2hc* mRNA expression as calculated as a ratio of the *GAPDH* band.

Table I. Expression of *LAT1* protein in regenerated bone.

	Extracellular matrix	Osteoid	Osteoblast	Osteocyte
Control group				
1 week	++	++	++	++
2 weeks	++	+	++	++
4 weeks	+	+/-	+	+/-
8 weeks	+/-	-	+/-	-
Mixture group				
1 week	++	+++	+++	+++
2 weeks	++	+	++	++
4 weeks	+	+/-	+	+/-
8 weeks	+/-	-	+/-	-

Expression: +++ strong expression; ++ moderate expression; + weak expression; +/- doubtful expression; - no expression.

Table II. Expression of *4F2hc* protein in regenerated bone.

	Extracellular matrix	Osteoid	Osteoblast	Osteocyte
Control group				
1 week	+	+	+	+
2 weeks	+/-	+/-	+/-	+/-
4 weeks	-	-	-	+/-
8 weeks	+/-	-	-	-
Mixture group				
1 week	++	++	++	++
2 weeks	++	+	++/+	+
4 weeks	+	+/-	+	+/-
8 weeks	+/-	-	+/-	-

Expression: ++ moderate expression; + weak expression; +/- doubtful expression; - no expression.



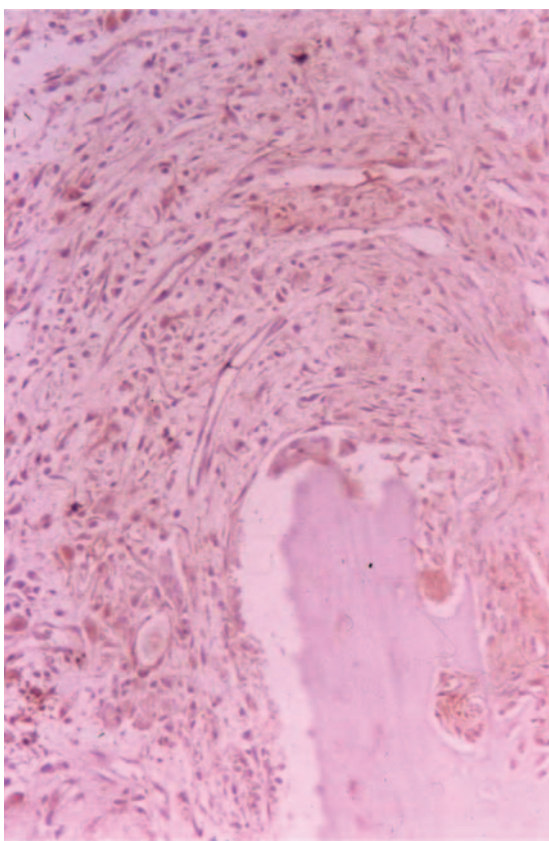


Figure 2. Expression of LAT1 protein in the control group (1 week). LAT1 was moderately expressed in osteoblasts and surrounding mesenchymal cells (x200).

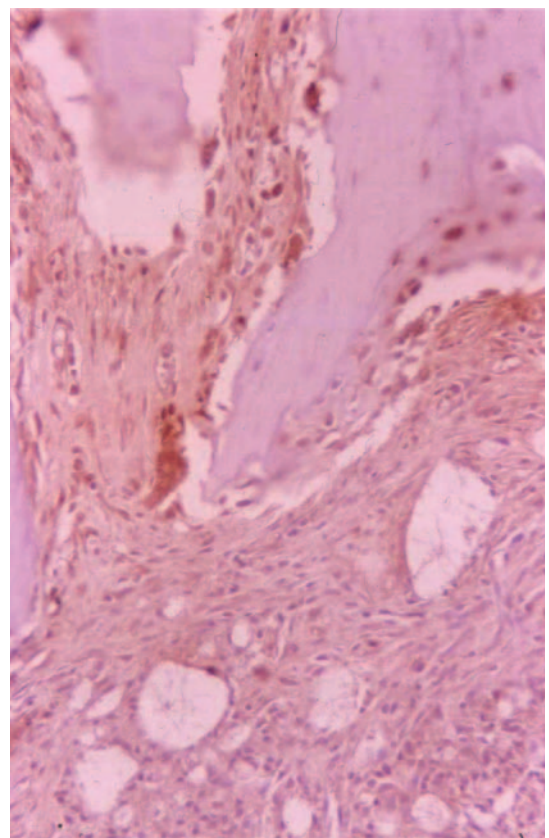


Figure 4. Expression of LAT1 protein in the tooth ash-plaster of Paris mixture group (1 week). LAT1 was highly expressed in osteoblasts and moderately expressed in surrounding mesenchymal cells around graft material (x200).

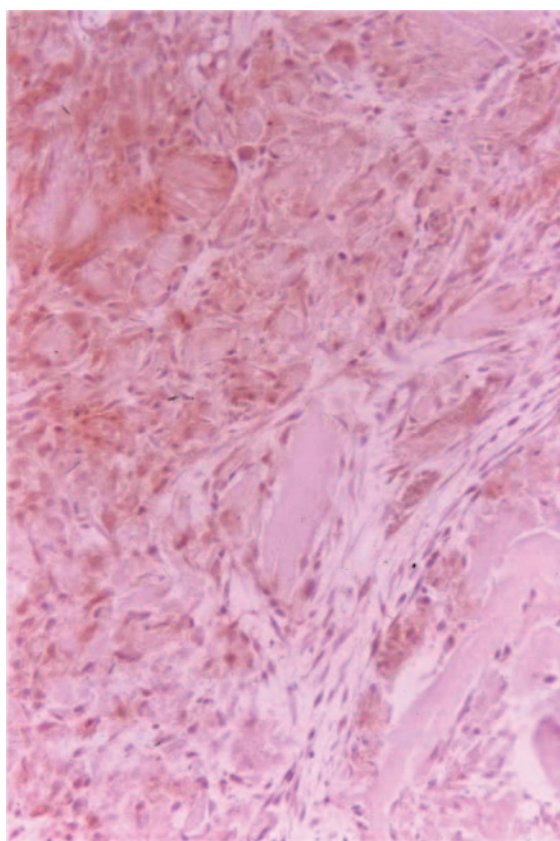


Figure 3. Expression of 4F2hc protein in the control group (1 week). The 4F2hc was minimally expressed in osteoblasts and mesenchymal cells (x200).

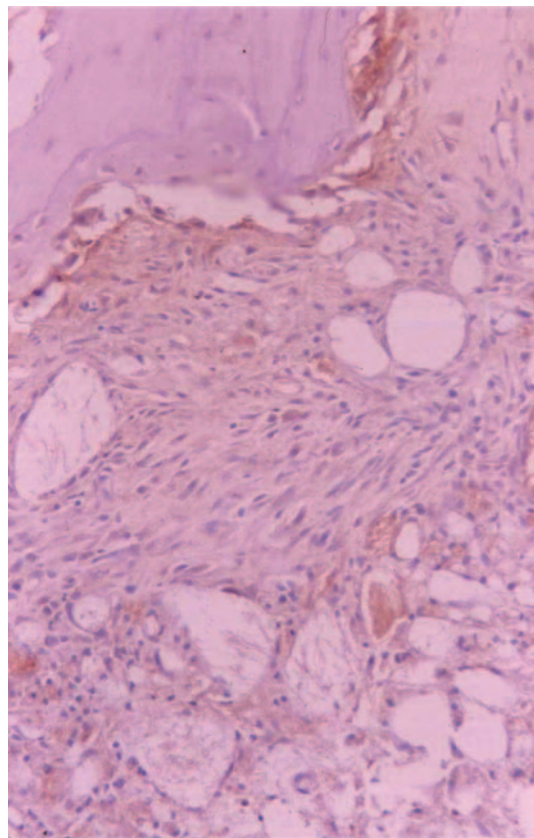


Figure 5. Expression of 4F2hc protein in the tooth ash-plaster of Paris mixture group (1 week). The 4F2hc was highly expressed in osteoblasts and moderately expressed in surrounding mesenchymal cells around graft materials. There was nonspecific expression in the graft material (x200).

(ii) *Mixture group*: In this group, the protein expression of LAT1 and 4F2hc showed a tendency to increase slightly compared to that of the control group at the early stage of implantation. LAT1 protein was strongly expressed around the new bone in the bone defect, and in osteoblasts and osteocytes in the pre-existing bone in the 1st week (Figure 4). It was moderately expressed in the interstitial tissue around the implant materials, but not in the implant itself. As time progressed, the expression of LAT1 seemed to decrease. 4F2hc protein was moderately expressed around the new bone, and in osteoblast and osteocytes in the pre-existing bone in the 1st week (Figure 5). The interstitial tissue around the implant had a similar level of expression. As with LAT1, the expression of 4F2hc protein decreased with time.

## Discussion

In the present study, so as to elucidate the role of LAT1 in the bone regeneration process, we investigated the expression pattern of LAT1 and its subunit 4F2hc in the healing process after implantation of a tooth ash-plaster of Paris mixture in calvarial osseous defected rats.

In the RT-PCR analysis, the LAT1 mRNA and its associated 4F2hc mRNA were detected at 1, 2, 4 and 8 weeks in both groups. The signals for the products of the LAT1 and 4F2hc were slightly stronger in the experimental group than in the control group. In both control and experimental groups, the signals for the products of the LAT1 and 4F2hc decreased after implantation in a time-dependent manner. LAT1 is highly expressed and plays an important role in growing and proliferating cells (12-14). As the results in this study correspond with those of previous reports (12-14), it is suggested that LAT1 and its subunit 4F2hc have an important role in supplying nutrition to cells at an early stage in the bone regeneration process, after the implantation of a tooth ash-plaster of Paris mixture. In order to investigate the protein expression patterns of LAT1 and 4F2hc in the healing process after the implantation of a tooth ash-plaster of Paris mixture the immunohistochemical analysis was performed.

After implantation, there was a tendency for the expression of LAT1 protein to increase slightly in the mixture group at the first week. At this time, it was highly expressed around the newly-formed bone in the bone defect. It also showed enhanced expression in interstitial tissue, but this decreased with time. After implantation, 4F2hc protein, which is a necessary factor for LAT1 function, seemed to increase slightly in the mixture group at the first, as well as second week. There was an enhanced expression around new bone and in internal osteoblast, osteocyte and interstitial tissue around the implant materials at the first week; however, the expression

decreased with time. It has been reported that LAT1 and 4F2hc protein expression increased as LAT1 has a key role in the early stage of oral cancer development (22). With our results in this study and a previous report (22), we suggest that LAT1 has a role: in transportation of neutral amino acids for protein biosynthesis in osteoblast and osteocyte around new bone; in the early stage of high-density new bone formation which is necessary in the osteoinduction and osteogenesis of bone graft materials. However, there was little difference in those expressions between the control group and the mixture group. An experiment may be needed to accurately quantitatively determine protein expression. In addition, it might be necessary to study: the expression of the neutral amino acid transporter, LAT1, after implantation of hetero bone (usually used in dental clinics as an implant); acidic or basic amino acid transporters; the early stage of post-implantation extensively using each implant.

In conclusion, LAT1, the neutral amino acid transporter, was highly expressed in osteoblasts and osteocytes around new bone at an early stage post implantation. These results showed that LAT1 is important for the transportation of neutral amino acids at the early stage of protein biosynthesis in high-density new bone formation and in the osteoinduction and osteogenesis of bone graft materials.

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