Differential Expression of System L Amino Acid Transporters during Wound Healing Process in the Skin of Young and Old Rats

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In order to elucidate the role of the system L-type amino acid transporters (LATs) in the wound healing process of aged and young subjects, we investigated the expression of LAT1, LAT2 and their subunit 4F2hc in the skin healing process after artificial wounds of dorsal skin in the young and old rats. Methods: The 1 cm full-thickness incisional wounds were made through the skin and panniculus carnosus muscle. The wounds were harvested at days 1, 3, 5 and 7 post-wounding, the experimental controls were harvested the skin of rat without wounds and the various analyses were performed. Results: In young rats, gradually and noticeable wound healing was detected, however, in old rats, wound healing was found to be greatly delayed. In young rats, the expression of LAT1 was increased rapidly on the day 1 after wound induction, on the other hand, in old rats, the expression of LAT1 after wound induction was not different from the control group. In young rats, the expression of LAT2 after the induction of wound was not different from the control group, however in old rats, the expression of LAT2 on the day 1 of wound induction was rapidly elevated. Conclusion: These results suggest that the LAT1 and LAT2 increase in the wound healing process after cell injury in young and old rats, respectively.

Key words amino acid; system L amino acid transporter; wound healing; aging; regeneration

Wound healing process could be considered to be a continuous morphological change of cells including the change of cell migration, adherence, *etc*.[1] Such changes of cells play decisive roles in other biological processes, for example, embryo development, tissue changes or tumor development.[1] Wound healing is a highly developed biological defense mechanism for the prevention of body fluid leakage, protection of the regenerating cellular barrier, and the removal of tissue residues and foreign materials, and it could be considered as a short term process inducing tissue regeneration and the removal of tissue debris for wound healing.[2—4] Also, the continuous growth and proliferation of cells are essential for the wound healing, 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.[5,6] Amino acid transport across the plasma membrane is mediated *via* amino acid transporters located on it.[5,6] 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.[7—9]

Recently, the system L-type amino acid transporters 1 and 2 (LAT1 and LAT2) were isolated.[7,8,10] They were predicted to be 12-membrane-spanning proteins that mediate Na^+-independent amino acid exchange.[7,8,10] They require an additional single-membrane-spanning protein, a heavy chain of 4F2 antigen (4F2hc), for their functional expression in the plasma membrane.[7,8,10—14] LAT1 mRNA is only expressed in restricted organs such as the brain, spleen, placenta and testis.[7,8,14,15] However, the mRNAs of LAT2 and 4F2hc are ubiquitously expressed in all normal embryonic and normal adult tissues.[7,8,10,14] In addition, LAT1 is highly expressed in malignant tumors presumably to support their continuous growth and proliferation.[7—9,16] The LAT1 prefers large neutral amino acids for its substrates,[7,8,17] while the LAT2 transports not only large neutral amino acids, but also small neutral amino acids, in a fashion that appears to have broader substrate selectivity than LAT1.[10,18—20]

It is reported that the system L-type amino acid transporters may be important in 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.[5,6] Thus, the system L may have an essential role in the wound healing process also. However, the expression and functional characterization of amino acid transporters, including the system L, in supplying nutrition to cells in the wound healing process are not known at all.

It is suggested that the age-related differences may be present in wound healing process.[21] Although the elderly can heal most wounds, they have a slower healing process, and impaired wound healing in the elderly presents a major clinical and economic problem.[21,22] With the aging population growing in both number and percentage, the importance of understanding the mechanisms underlying age-related impairments in healing is increased,[22] but the molecular mechanisms of wound healing in not only aged individuals but also young subjects have not been clearly determined. Furthermore, there are not any studies that the expression and

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functional characterization of amino acid transporters, including the system L, in supplying nutrition to cells and tissues in the wound healing process of aged and young subjects are examined and compared.

In the present study, therefore, in order to elucidate the role of the system L-type amino acid transporters in the wound healing process of aged and young subjects, we investigated the expression of LAT1, LAT2 and their subunit 4F2hc in the skin healing process after artificial wounds of dorsal skin in the young and old rats.

MATERIALS AND METHODS

Materials Affinity-purified anti-LAT1, anti-LAT2 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, U.S.A.).

Wound Healing Experiments This study was approved by the Animal Research Committee of Chosun University. Four-week old male Sprague-Dawley rats and Sprague-Dawley rats from one year up were selected for the young and old experimental samples in this study, respectively. 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*.

Skin wound was induced under ether anesthesia. The dorsum was shaved and sterilized according to conventional methods. Four equidistant 1 cm full-thickness incisional wounds were made through the skin and panniculus carnosus muscle, and left to heal by secondary intention. Intramuscular injection of 5 mg/kg gentamicin (Daesung Microbiological Labs Co., Gyunggi, Korea) was performed to prevent infection after surgery. The wounds were harvested at days 1, 3, 5 and 7 post-wounding and the experimental controls were harvested the skin of rat without wounds. The samples were bisected for histology and for RNA analysis (snap-frozen in liquid nitrogen).

Histological analysis and wound area measurement were performed as described elsewhere.[23)]

Real-Time Quantitative RT-PCR Analysis For real-time quantitative RT-PCR analysis, total RNA was prepared from each tissue using an RNA preparation kit (Isogen, Nippon-Gene, Japan) following the manufacturer's instructions. First strand cDNA was produced using SuperScript First-Strand Synthesis System for RT-PCR (Life Technologies Inc.) with an oligo dT primer. Two gram of total RNA was used for a reverse transcription reaction (20 μl).

The real-time PCR analysis was performed on an Applied Biosystems Prism 7900 Sequence Detection System (Applied Biosystems). Real-time quantitative PCR was performed in triplicate in 384-well plates; each 20 μl reaction consisted of 10 μl of SYBR Green Master Mix (Qiagen), 1 μl of 10 pmol/μl forward and reverse primers of LAT1, LAT2, 4F2hc and GAPDH. RT-PCR cycle parameters were 95 °C for 10 min, followed by 40 cycles at 95 °C for 30 s and 60 °C for 30 s, 72 °C for 30 s. Each of the 384-well real-time quantitative PCR plates included serial dilutions (1, 1/2, 1/4 and 1/8) of cDNA, which were used to generate relative standard curves for LAT1, LAT2, 4F2hc and GAPDH. We then converted real-time PCR cycle numbers to gene amounts (ng) on the basis of the equation.

For LAT1, the forward and reverse primers were 5′-GTC CCT CAA AGG TCA GGT GT-3′ and 5′-CAC CTC ACA GTG GCT GCT AT-3′, respectively. For LAT2, the forward and reverse primers were 5′-CGG AGT AGC CCT GAA GAA AG-3′ and 5′-TCC AGA CAA TGA GAG CAA GG-3′, respectively. For 4F2hc, the forward and reverse primers were 5′-CCC AAC TAT AAG GGC CAG AA-3′ and 5′-CCT GCA ACC AAG AAC TCA GA-3′, respectively.

Immunohistochemistry To investigate the expression pattern of LAT1, LAT2 and their subunit 4F2hc in the skin healing process, immunohistochemical analyses were performed as described elsewhere.[24,25)] The fixed tissues were incubated with anti-LAT1 (1 : 100 dilution), anti-LAT2 (1 : 100 dilution) or anti-4F2hc (1 : 100 dilution) affinity-purified primary antibody.

RESULTS

Histological Analysis and Morphometric Analysis of Skin Wound Healing in Young and Old Rats In young rats, until the last day of observation, the day 7, gradual would repair was detected, in contrast, in old rats, the recovery of the wound area was still not achieved (data not shown).

The result of hematoxylin and eosin (H & E) staining of wound tissues of young and old rats obtained at each time period was shown in Fig. 1A. Until the day 1, in both experiment groups, abundant tissue debris remained in the wound area. In young rats, wound closure was markedly progressed from the day 5, but in old rats, it was progressed gradually to the day 5 slowly. The regeneration and migration of epithelial cells in young rats were progressed noticeably before the day 5, however in old rats, it was not observed at the same time (Fig. 1A). The result of the recovery of wound area measured by morphometric methods was shown in Fig. 1B. From the day 1 of wound induction, the wound area of young rats was decreased noticeably in comparison with old rats, and on the day 7, very small size wounds were detected. However, in old rats, the trend of wound healing from the day 1 to the day 7 was very weak.

Expression of LAT1, LAT2 and 4F2hc mRNAs (Real-Time Quantitative RT-PCR) Wounds were induced in the dorsal skin of young rats and old rats, and to quantitate the expression of LAT1 and LAT2 and their subunit 4F2hc mRNAs according to would healing, Real-Time Quantitative RT-PCR analysis was performed using their primers. Figure 2 shows the amplification plot curve of the sample of each group as the result of Real-Time Quantitative RT-PCR analysis. In young rats, the expression of LAT1 mRNA was increased rapidly on the day 1 by 3.5 times higher than the control group and it was decreased on the day 3 in comparison with the day 1, however, it was increased 2 times higher than the control group (Fig. 2A). In old rats, the expression of LAT1 mRNA on the day 1 and the day 3 after the induction of wound was not different from the control group (Fig. 2A). In young rats, the expression of LAT2 mRNA on the day 1 and the day 3 after the induction of wound was not different from the control group (Fig. 2B). In contrast, in old rats, the expression of LAT2 mRNA increased rapidly on the day 1 after the induction of wound by 6 times higher than the

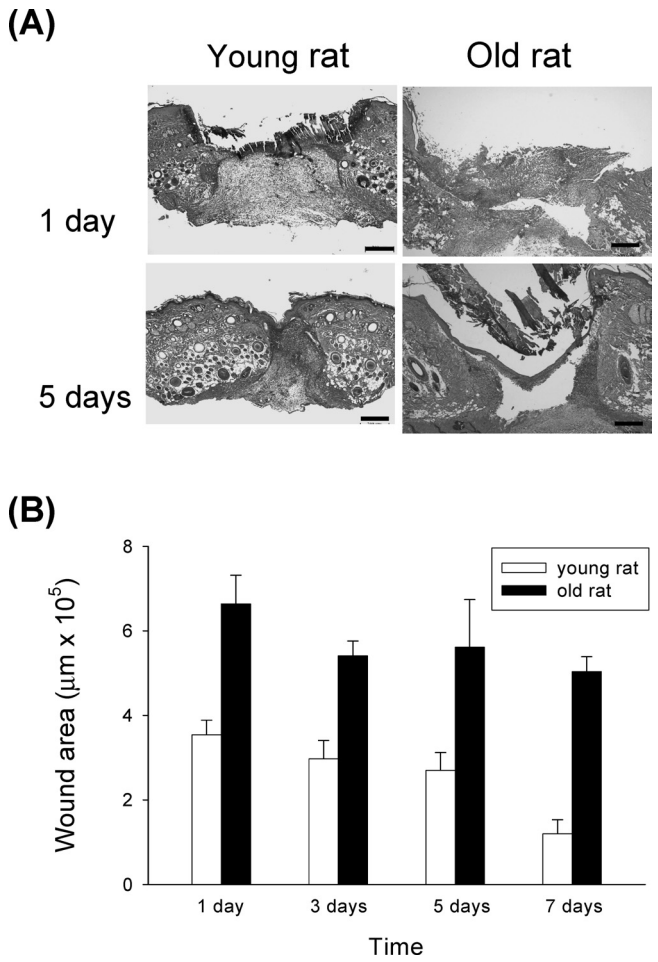

Fig. 1. Comparison of Healing Pattern and Wound Area after Artificial Wounds of Dorsal Skin in the Young and Old Rats

(A) The skin samples were stained with hematoxylin and eosin. All scale bars indicate 200 μm. (B) Wound areas were significantly greater in the old rats compared to the young rats at all days. Results represent means±S.E.M. (n=3—5 for each group).

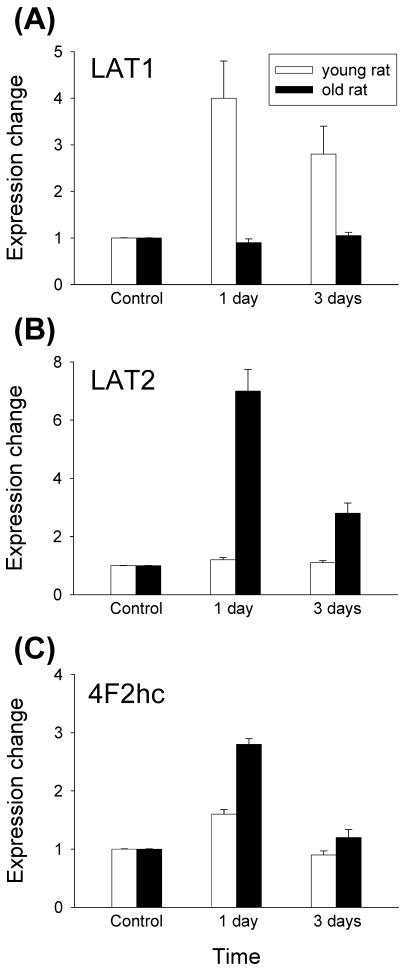

Fig. 2. Real-Time Quantitative RT-PCR of LAT1, LAT2 and 4F2hc Expressions

Expression levels for LAT1, LAT2 and 4F2hc were estimated using florescent quantitative RT-PCR in triplicate. The (A), (B) and (C) described the mRNA expressions of LAT1, LAT2 and 4F2hc, respectively.

control group and on the day 3, it was decreased in comparison with the day 1, nonetheless, it was increased 2 times higher than the control group (Fig. 2B). The expression of 4F2hc mRNA on the day 1 after the induction of wound in young rats was increased approximately 1.5 times and approximately 2.5 times in old rats in comparison with the control group, and on the day 3 after wound induction, the expression of 4F2hc similar to the control group was shown (Fig. 2C).

Immunohistochemistry After the induction of skin wound, to examine the protein expression level of LAT1, LAT2 and 4F2hc according to wound healing, immunohistochemical staining was performed using corresponding antibodies.

In young rats, regarding LAT1 protein, in epithelial tissues regenerated on the day 1 after wound induction, strong immune reaction was detected on cells in the upper area except the basal area (Fig. 3A). In the epithelial cell layer, on the day 3, immune reaction of cells migrating to the wound area was observed (data not shown). In old rat cases, on the day 1 after wound induction, the expression of LAT1 protein was very weak in epidermal and dermal tissues (Fig. 3B).

In young rats, partial expression of LAT2 protein was detected in wound tissues and histocytes in the dermis on the day 3 after wound induction, nevertheless, it was not expressed in the epidermis and other areas (Fig. 3C). However, on the day 3 tissues, in the regenerated epithelial tissues in the wound area, the expression of LAT2 protein was observed (Fig. 3D). In addition, on the day 1 after wound induction, in the epidermal tissues adjacent to the wound, the strong expression of LAT2 was detected (Fig. 3E).

In young and old rats, the expression of 4F2hc protein was slightly increased in migrating epithelial cells on the day 1 after wound induction (Figs. 3F, G)

DISCUSSION

In our study, the wound 1 cm in length constantly was generated in the skin of young and old rats, and the expression pattern and role of the amino acid transporters LAT1 and LAT2 were analyzed and compared during the wound healing process.

Based on the result of the histological and morphometric analyses (Fig. 1) in this study, it could be confirmed that wound healing was markedly more rapidly progressed in young rat cases than old rats. To examine whether such wound healing process of young and old rats is associated

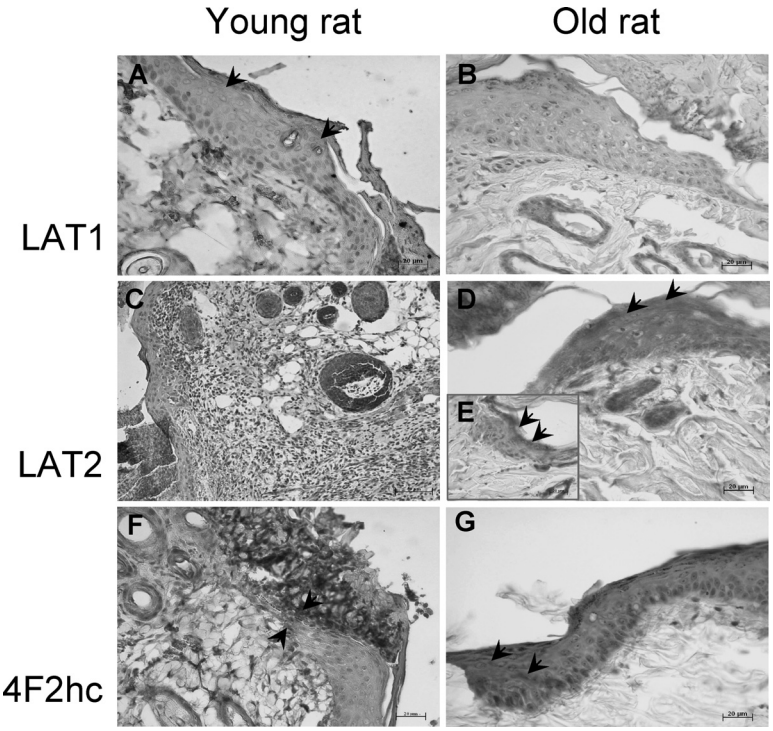

Fig. 3. Immunoreaction of LAT1, LAT2 and 4F2hc during Wound Healing after Artificial Wounds

Immunohistochemistry was performed after artificial wounds in young and old rats as described in Materials and Methods. All scale bars indicate 20 μm. Arrows indicate the expression of each protein. The (A) and (B) present the expression of LAT1 protein on the day 1 after wound induction in young and old rats, respectively. The (C) and (D) present the expression of LAT2 protein on the day 3 after wound induction in young and old rats, respectively. The (E) presents the expression of LAT2 protein on the day 1 after wound induction in old rats. The (F) and (G) present the expression of 4F2hc protein on the day 1 after wound induction in young and old rats, respectively.

with the amino acid transporters LAT1 and LAT2, the expression of the LAT1, LAT2 and their cofactor 4F2hc mRNAs was confirmed by real-time quantitative RT-PCR.

In real-time quantitative RT-PCR (Fig. 2), the expressions of LAT1 and LAT2 mRNAs were increased rapidly on the day 1 after wound induction in young and old rats, respectively. Clark R. A. F.,[26] and Mutsaers S. E. *et al*.[27] have reported that after wound induction, expression of proteins such as vitronectin and fibrinogen that are involved in platelet coagulation and the homeostasis of the formation of blood clots were enhanced. Also, other researchers have reported that during the middle and late period of the reformation of matrix, the expression of various proteinases, suppression molecules, metalomatrix proteinases (MMPs) *etc*., and their inhibitors is elevated or balanced and thus contributes to wound healing.[28,29] Such results reported by previous investigators suggest that proteins playing important roles in each wound healing period are different,[26,27] and such results imply that according to ages also, proteins playing important roles may be different from each other. With our results in this study and previous reports,[26—29] it is suggested that during the wound healing process after tissue injury, the neutral amino acid transporters LAT1 and LAT2 play the important roles during the early stage of wound healing in young and old rats, respectively.

To confirm the real-time quantitative RT-PCR results, the expression level and expression area of the LAT1, LAT2 and 4F2hc proteins according to wound healing were examined by immunohistochemical staining. In the immunohistochemical results (Fig. 3) of our study, LAT1 was strongly expressed primarily on migrating epithelial cells in young rats, which suggests that the LAT1 may be involved in the early phase of wound healing in young rats primarily. In old rats, LAT2 protein was strongly expressed primarily in the epithelial tissues next to the wound and regenerated epithelial tissues, which suggests that the LAT2 is involved in the wound healing of old rats primarily. Taken together, these results suggest that the increased LAT1 expression may significantly effect to wound healing process in young rats because of its high affinity for uptake of amino acid substrates.[8,17] In contrast, in old rats, even though the LAT2 was increased after wound induction, the wound healing is delayed because of lower than that of LAT1.[10,18,19]

It has been known that the 4F2hc plays an important role for the functional expression of LAT1 and LAT2 as a cofactor.[30] In this study, the levels of 4F2hc mRNA and protein were slightly increased after wound induction in young and old rats (Fig. 2C, Figs. 3F, G). The increased 4F2hc expression suggests that specific expression of LAT1 or LAT2 by wound induction involved in wound healing recovery processing with increasing 4F2hc as a cofactor.

Among the amino acid transport system, the system L-type amino acid transporter that is a Na^+-independent and responsible for the transport role of neutral amino acids including several essential amino acids.[5,6] The amino acid transport system L consists of the subtypes, LAT1 and LAT2, and their expression pattern is different depending on cells types, the environment and condition of cells.[8—10,16,18,19] In normal tissues, the area expressing LAT1 is limited to the brain, the placenta, the testis, *etc*., and it is overexpressed in malignant tumors primarily.[7,8] However, the LAT2 is ubiquitously expressed in all normal embryonic and normal adult tis-

sues.[10,18,19] Therefore, the results of our study suggest that during the wound healing process, the LAT1 sensitive to environmental change plays a very important role in the wound healing of young rats, and the LAT2 plays an important role in the wound healing of old rats. The roles of LAT1 and LAT2 in wound healing are considered as the tasks to be investigated more, nonetheless, in our study, during the wound healing process according to age, the neutral amino acid transport system L was found to be expressed differently form each other, which could be considered to be meaningful.

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