Regenerative Therapy 8 (2018) 1–8

Contents lists available at ScienceDirect

Regenerative Therapy

journal homepage: http://www.elsevier.com/locate/reth

Original Article

Electron microscopic observation of human auricular chondrocytes transplanted into peritoneal cavity of nude mice for cartilage regeneration

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ARTICLE INFO

Article history:
Received 13 October 2017
Received in revised form
10 November 2017
Accepted 13 November 2017

Keywords:
Chondrocytes
Macrophage
Lymphocyte
Endothelial cells
Peritoneal cavity
Electron microscopy

ABSTRACT

Restoration of damaged cartilage tissue has been deemed futile with current treatments. Although there have been many studies on cartilage regeneration thus far, there is no report that chondrocytes were completely re-differentiated in vitro. The clarification of cellular composition and matrix production during cartilage regeneration must be elucidated to fabricate viable mature cartilage in vitro. In order to achieve this aim, the chondrocytes cultured on coverslips were transplanted into the peritoneal cavities of mice. At different time points post-transplantation, the cartilage maturation progression and cells composing the regeneration were examined. Cartilage regeneration was confirmed by hematoxylin & eosin (HE) and toluidine blue staining. The maturation progression was carefully examined further by scanning electron microscopy (SEM) and transmission electron microscopy (TEM). At the first and second week time points, various cell shapes were observed using SEM. Chronologically, by the third week, the number of fibers increased, suggesting the progression of extracellular matrix (ECM) maturation. Observation through TEM revealed the chondrocytes located in close proximity to various cells including macrophage-like cells. On the second week, infiltration of lymphocytes and capillary vessels were observed, and after the third week, the chondrocytes had matured and were abundantly releasing extracellular matrix. Chronological observation of transplanted chondrocytes by electron microscopy revealed maturation of chondrocytes and accumulation of matrix during the re-differentiation process. Emerging patterns of host-derived cells such as macrophage-like cells and subsequent appearance of lymphocytes-like cells and angiogenesis were documented, providing crucial context for the identification of the cells responsible for in vivo cartilage regeneration.

1. Introduction

Cartilage tissue is a promising target for regenerative medicine, because it is difficult to be repaired once damaged. With an abundant demand for the clinical application of regenerative cartilages, the topic has been one of great interest. In 1994, Brittberg et al. first reported autologous chondrocyte implantation (ACI) [1] as a viable reconstructive effort of cartilage, and since then, many have reported the success of this therapy to ameliorate knee defects [2,3]. The preparation of ACI involves the injection of cultured chondrocyte suspension, resulting in a soft consistency at the time of transplantation which is expected to harden as maturation progresses in vivo. In recent clinical studies, some groups performed dorsal nasal augmentation procedures using autologous cultured chondrocytes. One group reported a two-stage

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Peer review under responsibility of the Japanese Society for Regenerative Medicine.

https://doi.org/10.1016/j.reth.2017.11.002

transplantation method in which regenerative cartilage was injection-transplanted subcutaneously in the patient's lower abdomen, and the matured cartilage tissue was then removed and transplanted into the nose for successful nasal reconstruction [4]. However, the two-step surgery requires multiple operations, resulting in increased potential for complications and burdens for the patient. Hoshi et al. reported the transplantation of regenerative cartilage into a scaffold before implantation into the target site [5]. The scaffold technique circumvented the disadvantages associated with the two-step surgery method. Yet, the cartilage implant was not fully matured pre-transplantation, thus creating a potential for composition deformities such as volume changes after transplantation. To form the desired physiologically and structurally stable regenerative cartilage, the cartilage must be matured in vitro. There are a number of factors, not previously applied for in vitro chondrogenesis, that has been reported to be involved in the proliferation or re-differentiation process of chondrocytes, including bone morphologic protein-2 (BMP-2) [6–8], transforming growth factor-β (TGF-β) [9,10], insulin-like growth factor (IGF) [11,12], fibroblast growth factor (FGF) [13–15] and hypoxia inducible factor-1α (HIF-1α) [16].

Production of mature cartilage in vitro necessitates a thorough comprehension of cartilage regeneration in vivo. The involvement of the host cells in cartilage regeneration has been shown in several reports. Fujihara et al. [17] showed that the interactions between chondrocytes and macrophages may increase granulocyte-colony stimulating factor secretion by macrophages and induce the expression of Fas ligand (FasL) on chondrocytes. FasL in turn induces the apoptosis of macrophages and suppresses tissue reactions, promoting the maturation of tissue-engineered cartilage. Additionally, they reported that the constructs containing Mif+/+ chondrocytes showed greater accumulation of cartilage matrix on the second week after transplantation than those containing Mif−/− chondrocytes [18]. Takebe et al. [19] showed that transient vascularization is important for cartilage regeneration. With accumulating evidence for the involvement of host cells, there are still many unclear points such as the mode of appearance of host-derived cells and temporal changes in the interaction between host and donor cells.

Along with host-derived cells, chondrocytes can also change their morphology. Electron microscopy is a technique commonly used in the field of regenerative medicine with which cell morphology and minute structures in and around cells can be analyzed. This imaging technique was utilized in past studies to examine the influence of scaffolds or extracellular matrixes (ECM) on the morphology of chondrocytes [20–22]. S. Nürnberger and colleagues provided evidence for differing chondrocyte morphology of horse cartilage in matrix-associated autologous chondrocyte transplantation (MACT) compared to horse cartilage chondrocytes in a collagen scaffold [23]. However, there have not been any reports on the temporal changes of the chondrocyte morphology or association of chondrocytes with host cells after transplantation.

In this study, we transplanted human auricular chondrocytes into the peritoneal cavities of nude mice and examined the emerging host cells in detail using electron microscopy at several time points in the re-differentiation process of chondrocytes.

2. Materials and methods

2.1. Cell preparation

All procedures were approved by the Research Ethics Committee of the University of Tokyo Hospital (ethical permission number 622). Human auricular chondrocytes from microtia patients were obtained from NAGATA Microtia and Reconstructive Plastic Surgery Clinic (Saitama, Japan). The patients provided the excess cartilage tissue voluntarily and agreed to the informed consent prescribed by the ethics committee. Human auricular chondrocytes were collected from the cartilage tissues as previously described [24]. The soft tissues and perichondria were removed from the specimen using a scalpel and scissors, and the auricular cartilage was finely minced. Cartilage fragments were incubated in 0.3% collagenase solution for 18 h at 37 °C in a shaking water bath. The solution was filtered through a cell strainer (100 μm pore size, BD Falcon), and the filtrate containing the human auricular chondrocytes were centrifuged at 400 g for 5 min. Cells were seeded at a density of 2.0×10^5 cells/dish onto φ100 mm collagen Type I Coated dish (AGC Techno Glass Co., Ltd.), and cultured for 10 days in Dulbecco's Modified Eagle Medium: Nutrient Mixture F-12 (DMEM/F12; Sigma–Aldrich Co.) supplemented with 5% human serum (Sigma–Aldrich Co.), 100 ng/mL FGF-2 (Kaken Pharmaceutical Co, Ltd.), 5 μg/mL insulin (Novo Nordisk Pharma Ltd.) and 1% penicillin/streptomycin (Sigma–Aldrich Co.) (cartilage growth medium: HFI) at 37 °C in a humidified atmosphere containing 5% CO_2. Once the chondrocytes had reached complete confluency, the cells were collected from the dishes using Trypsin–EDTA (Sigma–Aldrich Co.), centrifuged, re-suspended in CELLBANKER (Nippon Zenyaku Kogyo Co., Ltd.), and stored at −80 °C.

2.2. Transplantation of chondrocytes cultured on the coverslips

Thermanox Coverslips (Thermo Scientific NuncTM) were cut into squares the size of 5 mm × 5 mm. Frozen stocks of P0 chondrocytes were thawed and cultured with HFI (described above) for a week. Then, a cover slip was placed on the bottom of a 48-well plate (Falcon), where 5×10^4 cells of P2 chondrocytes were seeded and cultured in HFI for a week.

The human chondrocytes along with the coverslip were transplanted into peritoneal cavities of 6-week-old male Balb/c nu/nu mice (CLEA Japan, Inc.). Five milliliters of DMEM/F12 (Sigma–Aldrich Co.) was injected into the peritoneal cavity, gently pumped in the abdomen for 3 min, and the washing solution was collected every week for 4 weeks. At specific time points, the coverslips were harvested and used for histological analyses. Experimental protocols were approved by the Institutional Animal Care and Use Committee of the University of Tokyo.

2.3. Hematoxylin & eosin (HE) and toluidine blue staining

The collected tissues were fixed with 4% paraformaldehyde at 4 °C overnight. The samples were dehydrated and embedded in paraffin. 5 μm thick sections were prepared and stained with HE and toluidine blue stainings. Images of each histological section were taken with the Kompaktes Fluoreszenz-Mikroskop HS-Modellreihe BZ-9000 and BZ-II Analyzer (KEYENCE Corp.).

2.4. Scanning electron microscopy (SEM) and transmission electron microscopy (TEM)

The tissue samples attached to the cover slips were harvested on week 1, 2, 3 and 4 after transplantation, fixed with 2% glutaraldehyde in phosphate buffered saline, and subsequently post-fixed in 2% osmium tetra-oxide for 2 h in an ice bath. Thereafter, the specimens were dehydrated in a graded ethanol series.

To prepare the sample for SEM, specimens were dried using t-butyl alcohol freeze-drying, coated by osmium plasma ion coater and observed by SEM66 (JEM-6320F, JEOL). For TEM, dehydrated specimens were embedded in the epoxy resin. Ultrathin sections were obtained using the ultramicrotome technique. The ultrathin sections were stained with uranyl acetate for 10 min and then

soaked in lead staining solution for 5 min before observation by TEM (JEM-1200 EX, JEOL).

2.5. Statistical analysis

Data were expressed as mean ± SD, and statistically analyzed using Student's t test. A value of $p < 0.05$ was used to indicate statistical significance.

3. Results

3.1. The regeneration of cartilage tissues by human auricular chondrocytes in the peritoneal cavity of mouse

P2 human auricular chondrocytes cultured on a coverslip until confluency were transplanted into the peritoneal cavities of nude mice, collected every week for 4 weeks, and histologically analyzed (Fig. 1). As indicated by toluidine blue staining, no metachromasia was observed. The cells on the coverslip were multilayered 1 week after transplantation. In the second week, the islet-like tissues thickened and metachromasic areas were increasingly detected each consecutive week. Under observation by HE staining between the second and third week, luminal structures were observed around a cartilaginous tissue (Fig. 1, second weeks, arrow), indicating capillary formation.

From these results, it was confirmed that chondrocytes on coverslips regenerate cartilage tissues in the peritoneal cavities of mice. The increasing intensity of metachromasia from the cartilage tissue indicated complete maturation of the cartilage by the end of 4 weeks. The host-derived cells that infiltrated around cartilage via newly-formed vessels may have potential to influence the proliferation and re-differentiation of chondrocytes directly or indirectly.

3.2. ECM maturation occurs 3 weeks after transplantation

The change in the ultrastructure of cartilage matrix during the proliferation and regeneration progression was examined by SEM of the recovered coverslip (Fig. 2). In the SEM image, cells with various shapes were observed in the first (Fig. 2a) and second weeks (Fig. 2b). Fibers were observed in the third week (Fig. 2c, arrow head), and on the fourth week, the fibers became fine without directionality, and filled with some materials and fine granules (Fig. 2d, asterisk). These results indicated ECM maturation at 3 weeks post-transplantation, and provided confirmation for the results of Fig. 1.

3.3. Endothelial cells and lymphocytes appear following macrophages

Under SEM observation, maturation of the ECM was detectable, and then TEM was utilized to clearly depict the cell types and morphological changes of the cells present. On the first week, several types of cells were detected, including macrophage-like cells marked by dense and engulfed nuclei (Fig. 3a, arrowhead), and chondrocytes, with some fibroblast-like characteristics with elongated cell processes (Fig. 3a, arrow) and others of which were swollen. Cell-to-cell contact was observed between chondrocytes and macrophage-like cells (Fig. 4a, arrow: macrophage), and amongst chondrocytes (Fig. 4b). The chondrocytes contained abundant rough endoplasmic reticulum (rER) (Fig. 4b). Interstitial fibrous matter appeared rough (Fig. 3a).

At the second week time point, the appearance of a number of non-chondrocyte cells (Fig. 3b) along with the formation of capillaries was recognized (Fig. 3b, asterisk), reconfirming the result of Fig. 1. Among the non-chondrocyte cells present were lymphocyte-like cells (Fig. 3b, arrow), and macrophage-like cells functioning to phagocytose cells (Fig. 4c). The lymphocyte-like cells marked by round shape and small cytosolic area had no observed cell-to-cell contact with the chondrocytes. The development of rER (Fig. 4d, closed circle), and the Golgi apparatus (Fig. 4d, arrowhead) were clearly confirmed in chondrocytes. In addition, dense collagen fibrils were observed in the vicinity of cells (Fig. 4d asterisk), suggesting the production of ECM.

By the third week, the number of non-chondrocyte cells had decreased (Fig. 3c), and the volume of the cytoplasm of the chondrocytes decreased, from that seen in the second weeks (Fig. 3c, and Fig. 4e). The fibrils in the ECM became fine with increased density. These tendencies were strengthened on the fourth week (Fig. 3d).

Unlike the lymphocyte-like cells, at the first and second week time points, cell-to-cell contact was observed between chondrocytes and macrophage-like cells (Fig. 4a), suggesting the possibility of intercellular signaling. After 3 weeks, such findings were reversed, characterized by extended intercellular distances and no cell-to-cell contact. Macrophage-like cells, lymphocyte-like cells and vascular endothelial cells appeared relatively early in the maturation process of cartilage, suggesting a greater weight on their contribution to influence factors associated with maturation.

3.4. Changes in numbers of chondrocytes, macrophage-like cells, lymphocyte-like cells and vessels

To further investigate the above results, approximately 10 TEM images were taken at different region of interest in each week's sample. Based on the results of Fig. 3, the cell type was identified, and the number of cells per field of view was counted for chondrocytes, macrophage-like cells and lymphocyte-like cells, as well as the number of capillaries. The number of chondrocytes significantly decreased after three weeks (Fig. 5a). More than two macrophage-like cells could be confirmed on average in 1 visual field from the first week to the second weeks (Fig. 5b). Only in the second weeks were lymphocyte-like cells and capillary vessels counted (Fig. 5c and d). These results suggested that macrophages, lymphocytes and vascular endothelial cells may have some influences on early regeneration process and cartilage organization.

4. Discussion

The present results provided insight into the involvement of host-derived cells in the process of cartilage regeneration, which are speculated to produce factors that promote chondrocyte maturation. Identification of those factors will lead to the establishment of a successful in vitro method for cartilage regeneration.

There are very few papers in which cartilage is regenerated in the peritoneal cavity. Use of the omentum as an in vivo culture environment offers an efficient and effective method for tissue engineering due to the omentum's high vascularity and rich glycosaminoglycan and protein deposits. In addition to the synovium, pleura and pericardium, the omentum and peritoneum are serous membrane structures that are composed from layers of mesothelium [25]. Transplantation of the cartilage grafts into the mesothelium enhanced the chondrocyte counts and volumes compared to pre-transplant measurements [26]. Transplantation of regenerative tissue into the greater omentum of the abdominal cavity is advantageous due to its ability to collect and maintain vascularization without damage to other tissues [27,28]. Favorable environment for cartilage regeneration can be maintained in the peritoneal cavity, compared with other tissues such as trachea and joints [26,29].

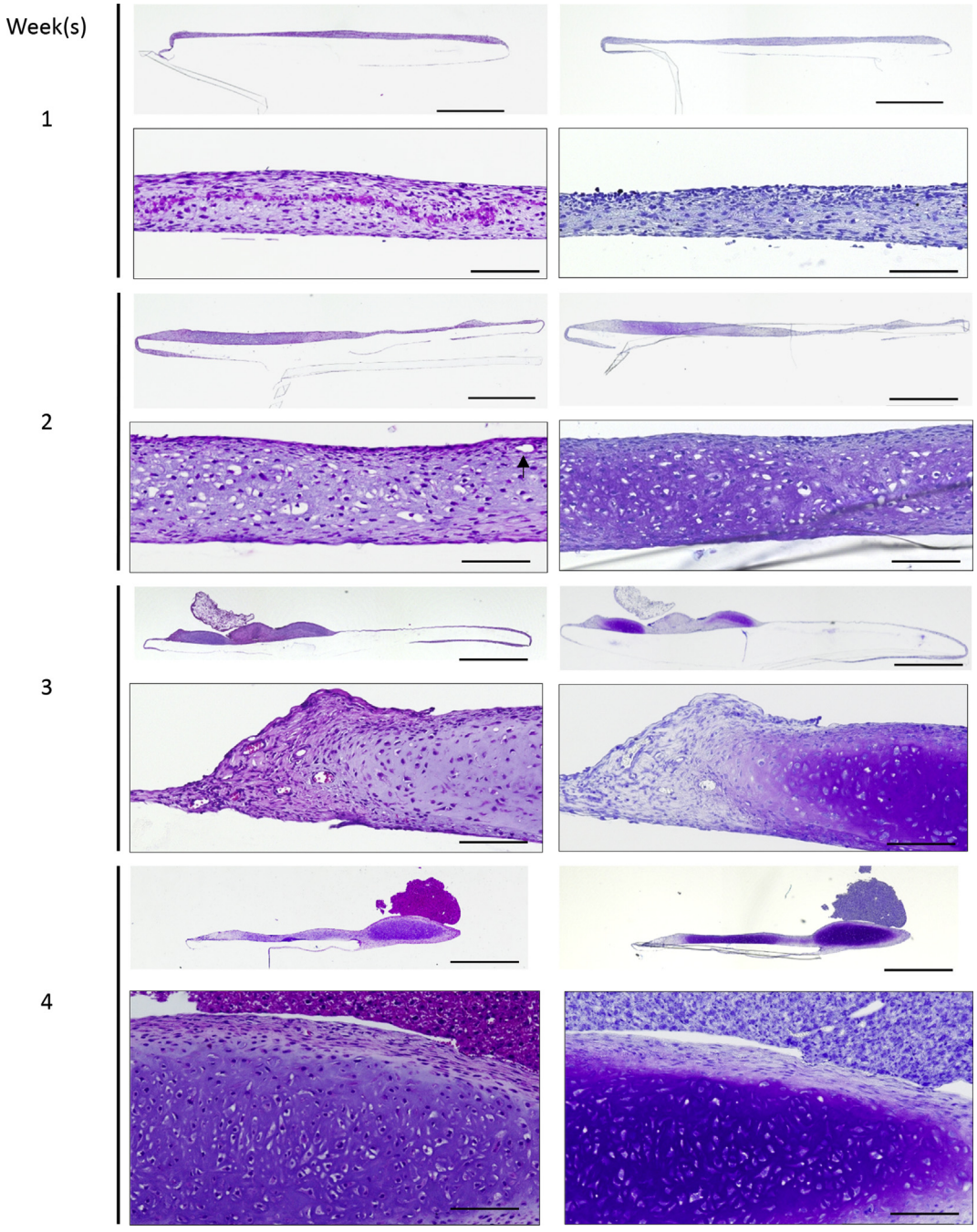

Fig. 1. Representative data of histological analysis of regenerative cartilage at different time points after transplantation of auricular chondrocytes with coverslips into the peritoneal cavity. (Left) Hematoxylin-Eosin (HE) staining (right) Toluidine blue staining. Low (the upper image in each panel) and high (lower) magnification images of each histological section are shown. The arrow head at the first week indicated host derived cells with small nuclear. The arrow (week 2, right lower panel) indicates capillary formation. Scale bar = 1000 μm (upper panel) and 200 μm (lower panel).

In addition to these clinical advantages, peritoneal cavity also has advantages in the basic sciences due to the ease at which cells and cytokines can be collected using a simple washing solution [30,31]. An examination of these harvested cells and cytokines at different time points can enable us to detail the timing of the emergence of host cells and cytokines influencing the cartilage regeneration process. It must be taken into account that coverslips transplanted into the peritoneal cavity may adhere to several different organs such as the liver, spleen, omentum, and intraperitoneal fat.

Although apparent tissue specificity of adhesion is unclear at present, it may be useful to compare the degree of cartilage regeneration in different adhesion sites. By analyzing the factors released by chondrocytes and host tissues, the tissues and factors that have the ability to enhance cartilage regeneration can be determined.

In this study, the appearance of inflammatory cells (e.g. macrophages) suggests its potential to have an influence on cartilage regeneration process. Although it is unclear whether the inflammatory cells such as macrophages reacted to the chondrocytes

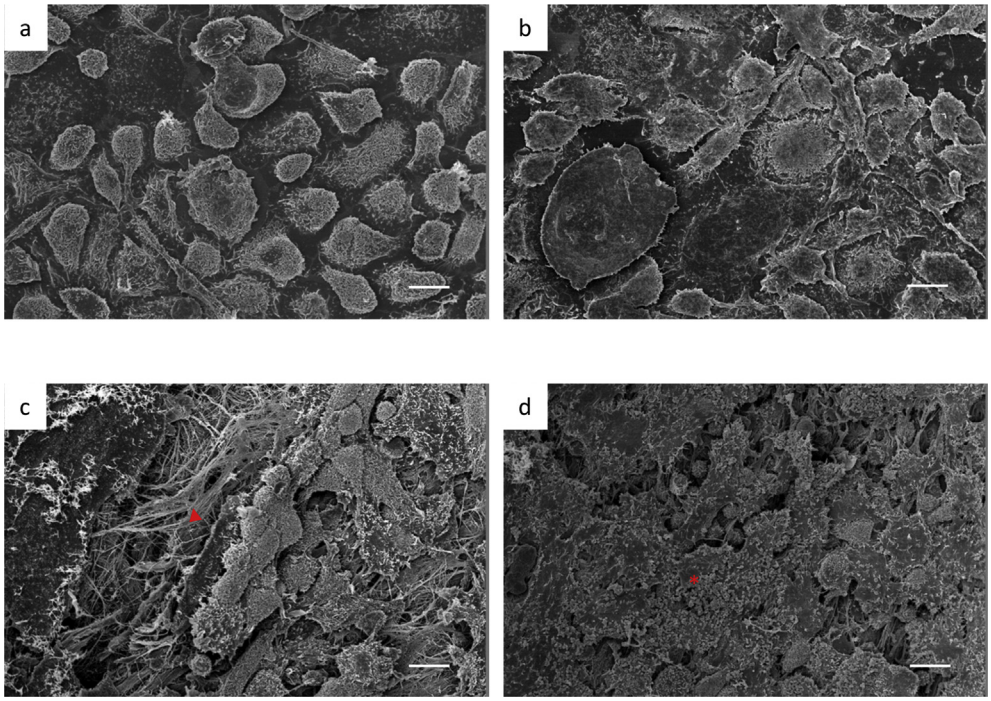

Fig. 2. Representative SEM images of chondrocytes on coverslip collected at week 1–4. a: first week, b: second weeks, c: third weeks, d: fourth weeks. The arrowhead (in c) indicates fibers. The asterisk (in d) indicates fine fibers filled with some materials and fine granules. Scale bar = 10 μm.

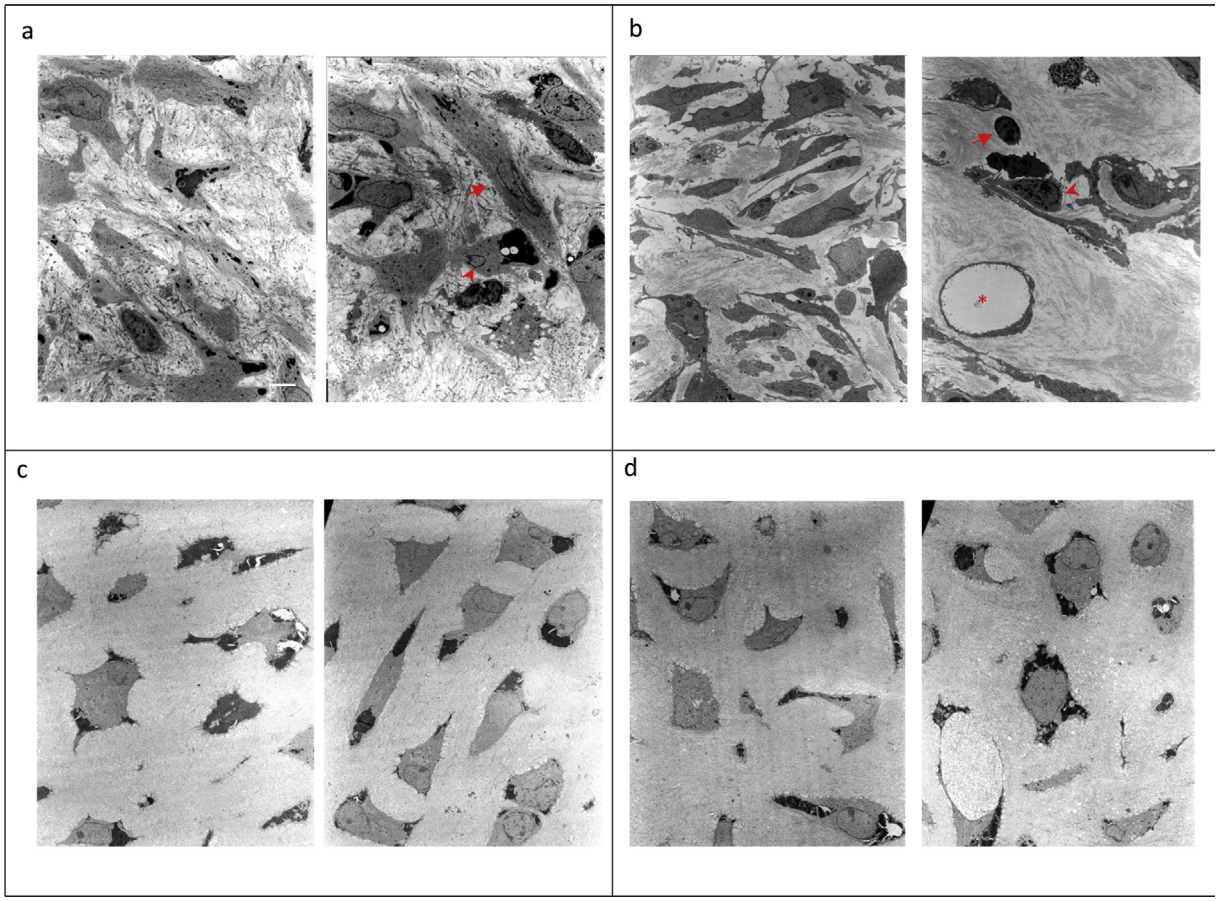

Fig. 3. Representative TEM images of chondrocytes on coverslip collected at week 1–4. a: first week, b: second weeks, c: third weeks, d: fourth weeks. In a, the arrowhead and the arrow indicate a macrophage-like cell and a chondrocyte, respectively. In b, the arrowhead, the arrow and the asterisk indicate a macrophage-like cell, lymphocyte-like cell and a capillary, respectively. Scale Bar = 5 μm.

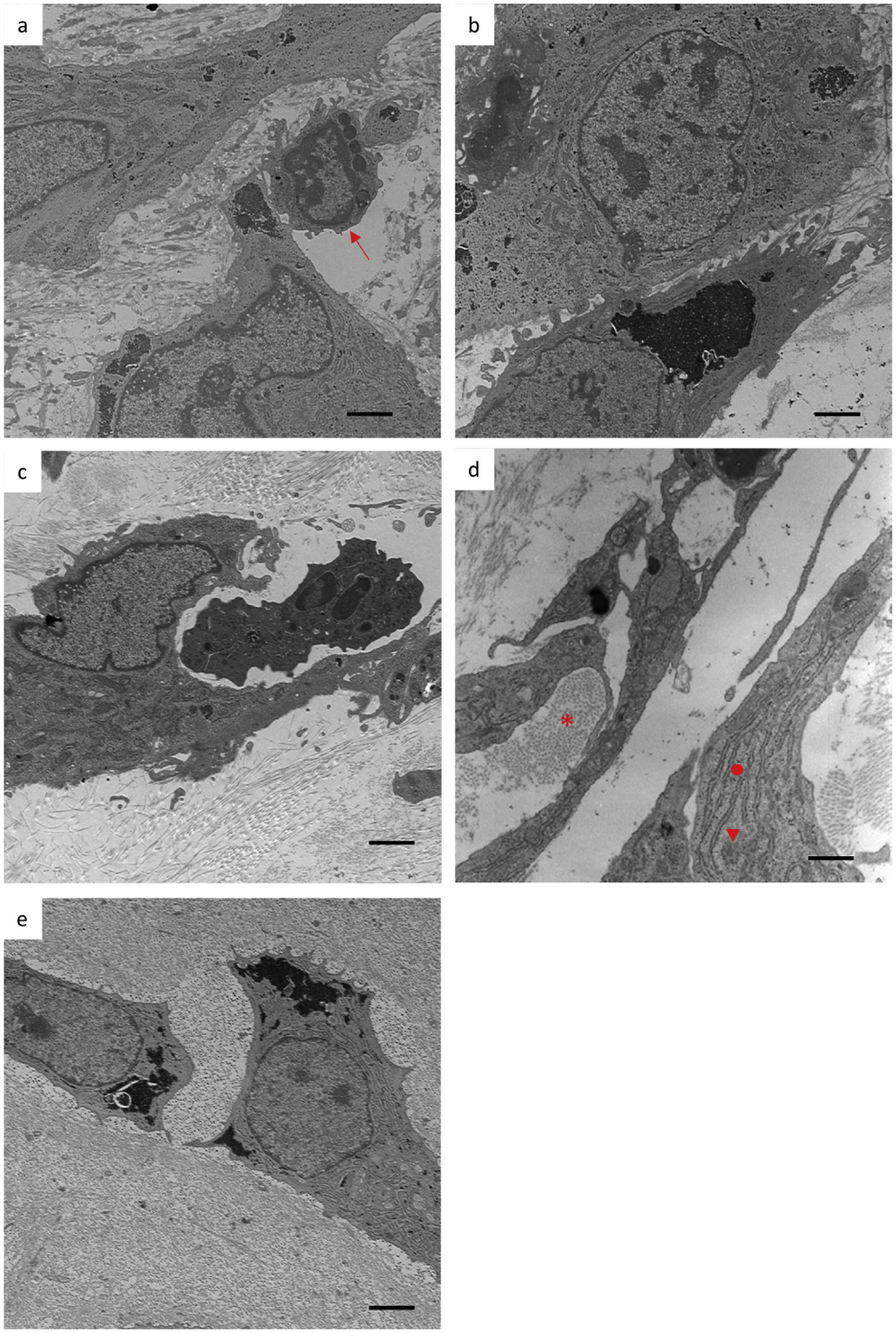

Fig. 4. Representative TEM images of chondrocytes on coverslip collected with high magnification. a, b: first week; c, d: second weeks; e: third weeks. In a, the arrow indicates a macrophage-like cell. In d, the closed circle, the arrowhead and the asterisk indicate rER, Golgi apparatus and dense collagen fibrils, respectively. a, b, c and e: scale bar = 2 μm; d: scale bar = 1 μm.

themselves or to the coverslip, it is still possible that macrophages have some influence on cartilage differentiation. Intercellular adhesion between chondrocytes and the host cells was confirmed relatively early after transplantation, which strongly supports the notion that host cells are affecting cartilage regeneration in the early phase. Our previous study suggested that the expression of FasL in chondrocytes induced the apoptosis of macrophages and suppressed tissue reactions, promoting the maturation of tissue-engineered cartilage [17]. This phenomenon may be applicable to this model as well. To detect the interaction of FasL and Fas between chondrocytes and macrophages, immunocytochemical examination using electron microscopy may be utilized in future studies.

HE staining revealed the initial formation of capillary vessels in the vicinity of chondrocytes, and subsequent accumulation of

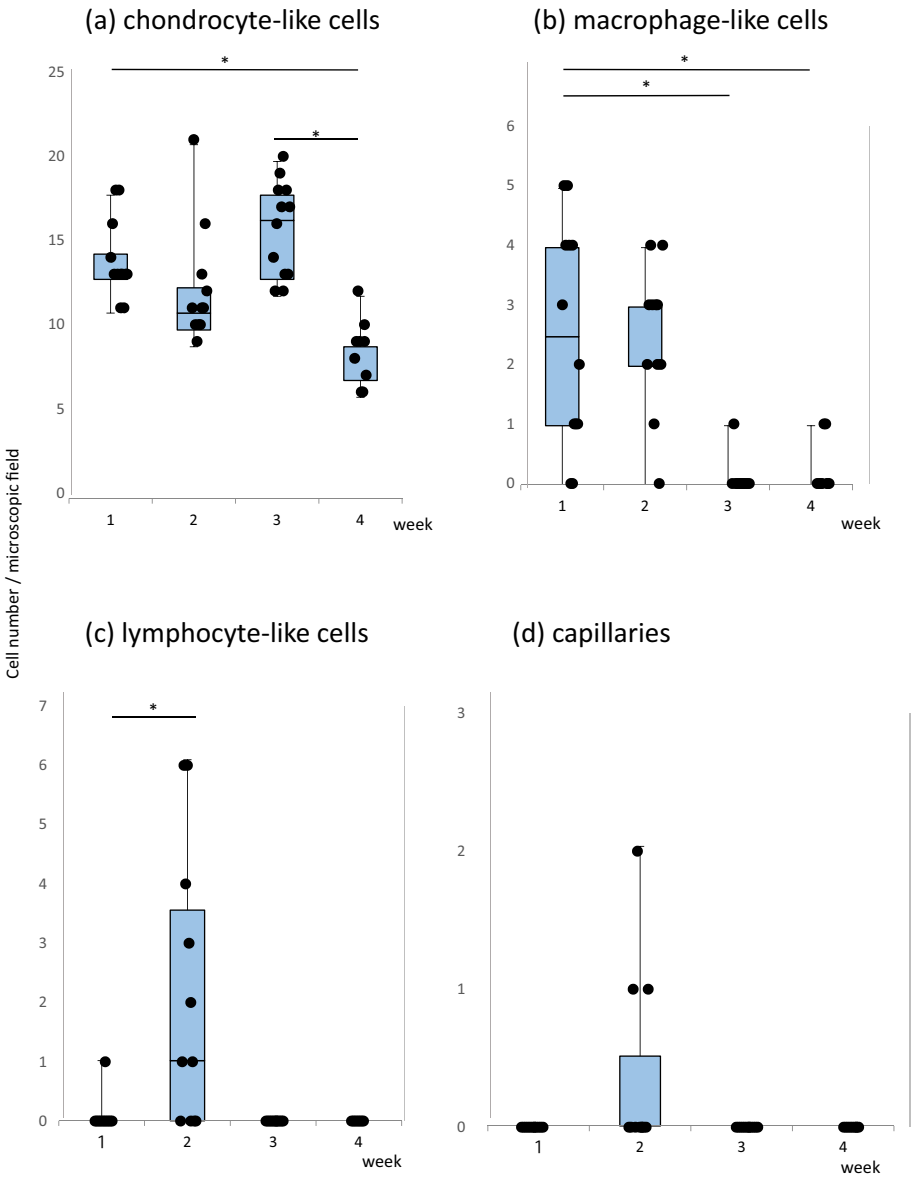

Fig. 5. Dot and box plots for the cell number per microscopic field of view. Each graph shows the number of chondrocyte-like cells (a), macrophage-like cells (b), lymphocyte-like cells (c), and capillaries (d) (n = 9–12). The dot represents cell numbers in each field of view. The both ends of the rectangle indicate the first quartile and third quartile, and the bar inside the rectangle represents the median. Bottom and top ends of the vertical lines indicate the minimum and maximum, respectively. * means $p < 0.05$.

capillaries after 3 weeks. While cartilage tissue itself is avascular, the vascularization of peripheral tissues can bring in immune-mediated macrophages and cytokines that aids in the progression of cartilage regeneration or destruction [32].

TEM imaging also revealed detailed morphological changes during cartilage regeneration. Chondrocytes had high cell densities in the first week and showed mixed populations of spindle shaped cells and swollen cells. By the second week, chondrocytes contained an abundance of rough endoplasmic reticulums and Golgi apparatuses. Extracellular matrix formation increased until the fourth week, which was confirmed by the toluidine blue staining.

Due to the narrow field of view used in electron microscopic observations, there are limitations to the breadth of information obtained from the entire specimen. Future studies could utilize other modalities such as flow-cytometry or immunostaining to analyze the cells attached to the chondrocytes on the cover slip.

5. Conclusion

By observing transplanted chondrocytes using electron microscopy sequentially, it was revealed that dramatic changes to the morphology and matrix accumulation during the redifferentiation of chondrocytes occurred between the second and third weeks post transplantation. We elucidated the chronological appearance pattern of macrophage-like cells, along with the subsequent appearance of lymphocyte-like cells and angiogenesis. This study implicates the important role that the host-derived cells play during in vivo cartilage regeneration.

Acknowledgments

We would like to thank Mr. Makoto Watanabe, Ms. Akiko Takakura, Ms. Dan Riu for their technical support, as well as Dr. Satoru Nagata for providing us with the human auricular cartilages

from his patients. This work was supported by Grants-in-Aid for Scientific Research from the Ministry of Education, Culture, Sports, Science and Technology of Japan (MEXT, 15H05040).

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