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(Citation)

Bulletin of health sciences Kobe, 17:23-33

(Issue Date)

2001-11-30

(Resource Type)

departmental bulletin paper

(Version)

Version of Record

(JaLCD0I)

<https://doi.org/10.24546/00098331>

(URL)

<https://hdl.handle.net/20.500.14094/00098331>



An immunohistochemical and immunoelectron microscopic study of beta integrins ($\alpha 4\beta 1$, $\alpha 5\beta 1$) and matrix metalloproteinases expression in rheumatoid synovial pannus formation in rheumatoid arthritis

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To investigate the mechanism of synovial pannus formation in rheumatoid arthritis, immunohistochemical and immunoelectron microscopic studies with monoclonal antibodies against the adhesion molecules, $\alpha 4\beta 1$, $\alpha 5\beta 1$ integrins and matrix metalloproteinases (MMP-1, MMP-3) and tissue inhibitor of metalloproteinase (TIMP-1), were carried out to determine the pattern of the distribution of these molecules at the rheumatoid synovial cartilage junction. Treatment with purified anti-human monoclonal antibodies, anti- $\alpha 4\beta 1$ and $\alpha 5\beta 1$ resulted in membrane staining of most of the cells infiltrating the synovial tissue and bordering the pannus cartilage junction. The $\alpha 4\beta 1$ and $\alpha 5\beta 1$ interaction appeared to be involved in the attachment of the pannus. The VLA-5 molecule was found in a pericellular and interterritorial matrix distribution in the present study. Staining for MMP-1 and MMP-3 was particularly intense at the pannus-cartilage junction. These molecules were also strongly stained in the cartilage matrix bordering the tip of pannus invasion. TIMP-1 staining disclosed very weak cellular staining and weak extracellular staining at the pannus-cartilage junction. The present study indicates that a receptor-ligand interaction between $\beta 1$ integrin and cartilage matrix may occur at the early stage of pannus formation, and that this is followed by elevated proteolytic activity of MMPs and a decreased level of TIMP-1, and in this way contributing to pannus invasion and cartilage destruction.

Key Words: Synovial-pannus formation, $\beta 1$ integrins, Matrix metalloproteinases (MMP-1, MMP-3), Tissue inhibitor of metalloproteinase (TIMP-1)

Introduction

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In rheumatoid arthritis, proliferating synovial cells penetrate the cartilage in the form of a pannus, and cartilage

destruction takes place in the zone of contact between the cells and the cartilage. In response to as yet unknown autocoids, in addition to the presence of immune complexes in the superficial cartilage^{1,2)}, the proliferating synovial tissue penetrates and degrades the cartilage. The mechanisms responsible for pannus formation are not fully understood, but there is general agreement as to the role of pannus, growing over the cartilage surface and invading the cartilage matrix, in the production of cartilage injury³⁻⁶⁾. In established disease, the pannus is commonly regarded as an extension of the synoviocytes of the joint lining layer, expanding under the influence of an immunologically mediated process⁶⁾, but little is unknown about when pannus first appears and its initial localization.

In a previous study, the authors demonstrated that recombinant human interleukin-1 (IL-1) stimulated monocyte and synovial cell attachment to rheumatoid cartilage *in vitro*⁷⁾. In this study, large numbers of monocytes from healthy individuals and cultured synovial cells were observed to attach to the rheumatoid articular surface in the presence of IL-1, suggesting that IL-1 generated by adherent monocytes and synovial cells may increase the binding of these cells to cartilage matrix protein. Furthermore, the cells attached to the cartilage surface strongly expressed $\alpha 4$ and $\alpha 5$ integrins. In another previous study, using immunohistochemical and immunoelectron microscopic techniques, and monoclonal antibodies against the $\beta 1$ integrin

adhesion molecules, we examined the pattern of distribution of these molecules at the rheumatoid synovial cartilage junction. In this study, we observed that $\alpha 3$, $\alpha 4$ and $\alpha 5$ integrins were the predominant $\beta 1$ integrins expressed by rheumatoid synovial pannus⁸⁾. Since these three integrins all function as fibronectin receptors⁹⁻¹¹⁾, it was suggested that the fibronectin-rich environment of the rheumatoid cartilage surface trapped pannus cells expressing high level of these molecules.

Synovial cell invasion of cartilage is thought to be a multi-step process involving the adhesion of synovial cells to extracellular cartilage matrix components, proteolytic degradation of the matrix, and invasion through the digested matrix. It has been concluded that, although integrins play an important role in normal cell growth and differentiation, increased expression of these agents on tumor cells has been correlated with aggressive invasive behavior of these cells¹²⁻¹⁴⁾. These observations indicate that both $\beta 1$ integrin-mediated cell migration and proteinase activity are required for cellular invasion. The expression of proteinases is controlled partly by direct signalling via $\beta 1$ integrins¹⁵⁾.

Matrix metalloproteinases (MMPs) are a family of enzymes that are secreted in zymogen form by connective tissue cells, inflammatory phagocytes, and a number of varieties of transformed cells¹⁵⁾. MMPs play an important role in various physiological changes such as tissue remodeling, morphogenesis and cellular invasion of extra-

cellular tissue^{12,16}). Immunostaining experiments have shown intense staining of collagenase (MMP-1) and stromelysin (MMP-3) at the pannus cartilage junction^{17,18}. These studies have indicated that components of the ternary MMP-1/ TIMP-1 (tissue inhibitor of metalloproteinase) /MMP-2 (gelatinase) complex are coexpressed in the extension of synovial lining cells to the hyaline articular cartilage where it leads to tissue destruction¹⁸. A recent study has shown that there is a striking decrease in the amount of TIMP-1 secreted by rheumatoid synovial membrane compared with normal synovium¹⁹. This has important implications for the pathogenesis of cartilage destruction and erosion in RA and has raised the possibility that the TIMP system is overwhelmed by massive amounts of metalloproteinase production in RA²⁰.

In the present study, an immunohistochemical investigation using immunoperoxidase staining methods has been carried out to determine more precisely whether $\beta 1$ integrin adhesion molecules expressed on pannus and cartilage, play a role in the proteolytic activation on MMP to enhance cellular invasion into cartilage matrix.

Materials and Methods

Thirty-one samples of rheumatoid articular cartilage covered with pannus from 31 patients were obtained during synovectomy or joint replacement surgery. All patients were considered to have moderate to severe active syno-

vitis at the time of surgery. All patients were rheumatoid factor positive and fulfilled the ACR diagnostic criteria for rheumatoid arthritis. Several samples of pannus-cartilage junction from each patient studied were selected to contain active pannus on the basis of naked eye examination, and this was confirmed by light microscopy using haematoxylin and eosin staining. Fibrous pannus was discarded on the basis of absence of cellularity. Each specimen was stained immediately after collection.

Purified anti-human monoclonal antibodies denoted as CDw49d ($\alpha 4 \beta 1$, VLA4) and CDw49e ($\alpha 5 \beta 1$, VLA-5) were obtained from Immunotech (Marseille, Cedex, France). These monoclonal antibodies had similar specific avidities for their antigen. Purified anti-human MMP-1²¹, anti-human MMP-3²² and anti-human TIMP-1²³ were purchased from Toyama Pharmaceutical Co (Toyama, Japan). Purified mouse IgG was obtained from Cappel Laboratories (Cochranville, Pa). Avidin biotinylated peroxidase (ABC-kit) was obtained from Vector Laboratories (Burlingame, Calif), and 3-3'-diaminobenzidine was purchased from Sigma Chemical Co (St. Louis, Mo). Frozen sections, about 4-6 μ m thick, were cut on a cryostat (Bright, Huntington, England) at -20°C , and mounted on gelatin and egg albumin coated slides. After drying at room temperature, the sections were washed with phosphate-buffered saline (PBS). Normal goat serum, diluted 1:200, was applied to the sections for 20 min. After washing,

they were incubated with 100-200 μ l of diluted monoclonal antibody for 60 min. After washing with PBS, biotinylated peroxidase conjugated goat anti-mouse IgG antibody (Becton-Dickinson Monoclonal Center, Mountainview, Calif.) was added. The tissue was then incubated with 3mg of 3-3'-diaminobenzidine in 10 ml of Tris HCl buffer, pH7.5, for 10 min. The specimens were then washed in PBS and dried at room temperature. Sections were stained with haematoxylin for background and nuclear staining of the cells. For electron microscopic examination, the sections were fixed with 1% osmium tetroxide(OsO₄) for 1h and washed in PBS and that was dehydrated in graded alcohol to 100%. While the sections were still wet, plastic capsules filled with Epon 812, the slides were heated on a hot plate and the sections were removed from slides. Fields were chosen for electron microscopic study by examining the light microscopic pictures on a dissecting microscope. Sections were cut on an LKB microtome and examined in a Nihon Densi electron microscope without counterstaining with lead citrate.

Results

A variety of cell types stained positively for $\alpha 4 \beta 1$ and $\alpha 5 \beta 1$ antibodies, including the synovial lining cells, mononuclear cells and endothelial cells of the post-capillary venules (PCV). The percentage of cells stained positively for different molecules at the pannus cartilage junction varied. The

reasons for such variation in the total cells counted were due to difference in specimen size and a wide variation in cellularity. Some degree of hyperplasia of the synovial lining cells was observed.

Beta 1 integrins

When specimens were treated with anti- $\alpha 4 \beta 1$ and anti- $\alpha 5 \beta 1$, most of the cells located at the cartilage border showed strong membrane staining with both antibodies (Figure 1). The $\alpha 5 \beta 1$ positive cells usually outnumbered the $\alpha 4 \beta 1$ positive cells. The percentage of total cells that stained with $\alpha 5 \beta 1$ in sections remained fairly constant at 50-75%, regardless of the numbers of various cell types present. There was strong anti- $\alpha 5 \beta 1$ staining on chondrocytes at or close to the pannus-cartilage junction. When the specimen was treated with anti-VLA5, most of the cells located at the cartilage border showed strong staining with this antibody. In the electron microscopic examination, the electron dense materials were observed in a patchy distribution on the cell membrane, and these materials were observed to be in contact with cartilage matrix (Figure 2).

Matrix metalloproteinase distribution in pannus cartilage junction

Staining for MMP-1 and MMP-3 was particularly intense at the pannus-cartilage junction. Similarly, at the synovial-pannus junction, the pannocytes were intensively positive for MMP-1 and MMP-3. These molecules were

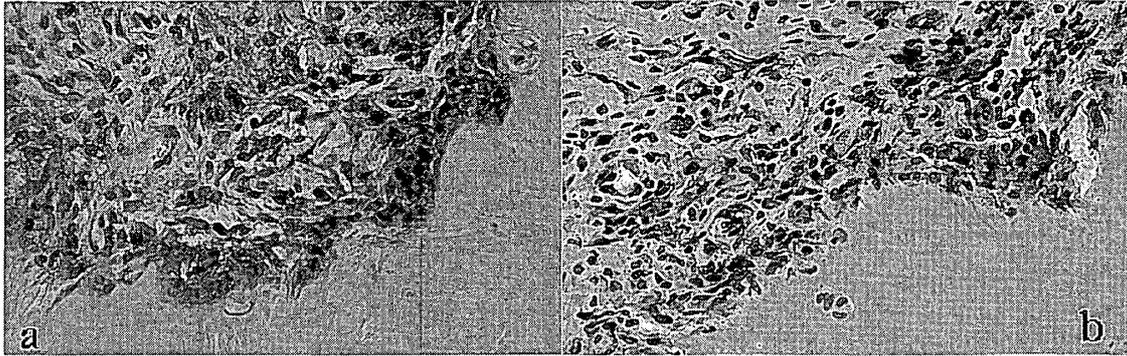


Figure 1. $\alpha 4 \beta 1$ and $\alpha 5 \beta 1$ staining of the rheumatoid synovial pannus-cartilage junction. Diffuse and weakly positive $\alpha 4 \beta 1$ (a) and strongly positive $\alpha 5 \beta 1$ fibroblastic cells and macrophages are observed at the pannus-cartilage border (b). Original magnification X200 (CA cartilage, PA pannus)

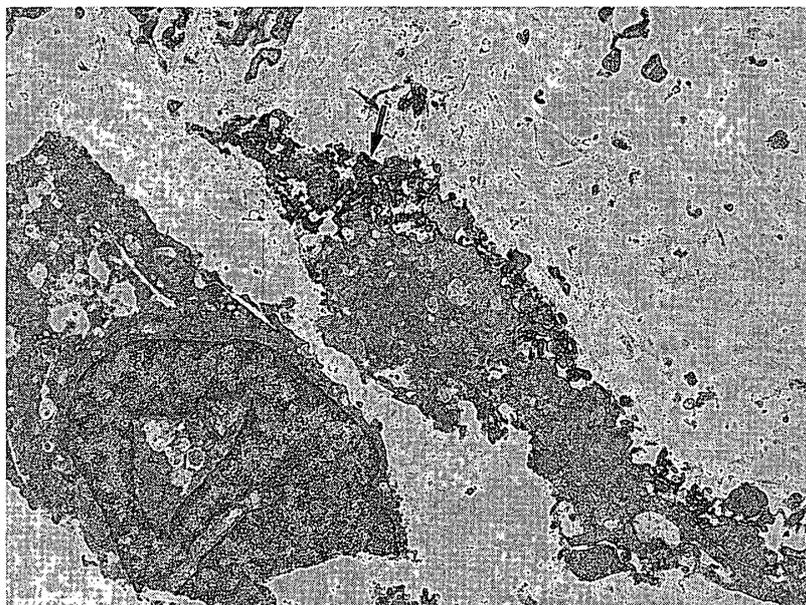


Figure 2. Electron microscopic findings of VLA-5 positive cells in the pannus cartilage border. Electron-dense materials are observed in a patchy distribution on the cell membrane. These materials are observed to be in contact with cartilage matrix. Arrow indicates the peroxidase-positive materials. The patchy staining on the cell membrane is indicated by arrows. Original magnification x5,000

also strongly stained in the interterritorial matrix of cartilage of the tip of pannus invasion (Figure 3a, 3b). In the electron microscopic examination, the electron dense materials were observed in a patchy distribution on the cell membrane infiltrated into the cartilage border. These materials were observed to be in contact with cartilage

matrix (Figure 4)

Tissue inhibitor-1 of matrix metalloproteinase (TIMP-1)

Although TIMP-1 staining was detectable in lining cells, there was an absence of TIMP-1 staining in the perivascular areas. There was very weak cellular staining and weak stain-

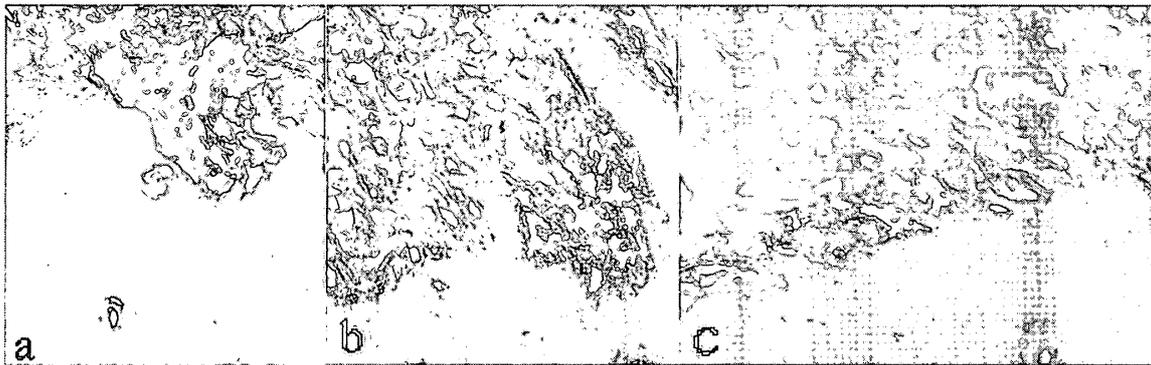


Figure 3. MMP-1, MMP-3 and TIMP-1 stainings of lining and sublining layer. Almost all cells of the lining layer showed strong MMP-1 (Figure 3a) and MMP-3 (Figure 3b) staining. TIMP-1 positive cells were usually stained very weakly and were not stained in the lining layer except in rare sections (Figure 3c). Original Magnification X 200

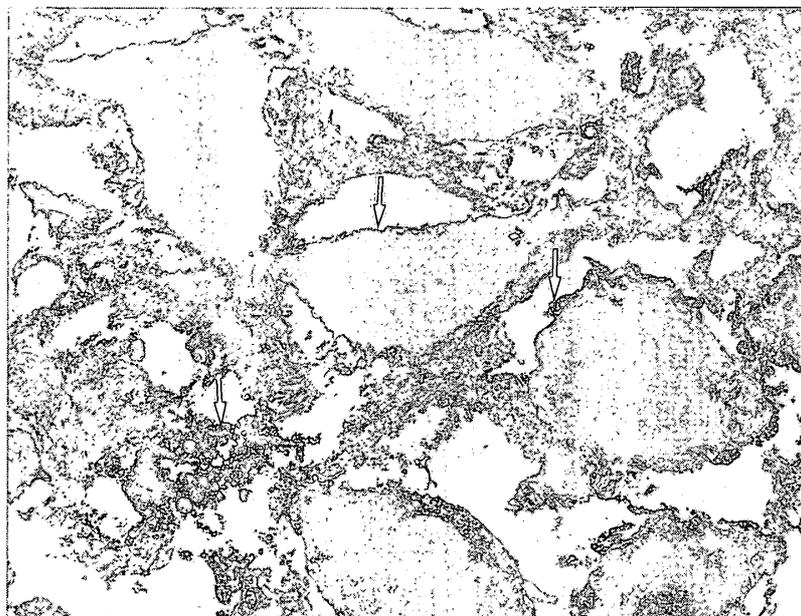


Figure 4. Electron microscopic appearance of MMP-1-positive cells at pannus-cartilage junction. The cell membrane of infiltrated into cartilage are observed to be in contact with cartilage matrix. Arrows indicate the peroxidase-positive products.

ing of extracellular TIMP-1 at the pannus cartilage junction (Figure 3c). In contrast to TIMP-1, MMP-1 and MMP-3 were strongly expressed in the cells of the invasive pannus tissue. The cells of the pannus and the interface of the synovial-cartilage junction were weakly positive or negative.

Discussion

Although the rheumatoid pannus is characterized by increased fibroblast proliferation, the initial triggering factors contributing to pannus formation are still unclear²⁴⁻²⁷. Synovial cell invasion of articular cartilage is a multi-step process, involving the adhesion of

synovial cell to extracellular matrix (ECM) components, proteolytic degradation of the matrix, and invasion through the digested matrix. In a previous study, we have reported that binding of monocytes and synovial cells to cartilage matrix was increased in the presence of IL-1⁷⁾. Synovial cell attachment to cartilage may be the initial step in pannus formation. In another study, we showed that $\alpha 4 \beta 1$ and $\alpha 5 \beta 1$ integrins are the predominant $\beta 1$ integrins expressed by rheumatoid synovial pannus⁸⁾. In this study, we suggested that receptor-ligand interaction between $\alpha 5 \beta 1$ and cartilage matrix may occur in the early stages of pannus formation. Furthermore, we emphasized that an increase in $\beta 1$ integrin may be necessary for the growth of the pannus and also for the upregulation of the VLA molecules, leading secondarily to increased attachment.

Most of the ECM integrins include a $\beta 1$ subunit, which can be combined with any one of the $\alpha 1$ - $\alpha 7$ chains. Recently, it has been shown that treatment of glioma cells with an antibody to $\alpha 5$ increased their invasiveness significantly²⁸⁾. This suggests that when the synovial tissue comes in contact with an appropriate ECM molecule, there may be an increased expression of integrins on the cell surface that would facilitate the invasion process. In the present study, we have therefore, investigated the morphological character and distribution of cells expressing $\alpha 4 \beta 1$, $\alpha 5 \beta 1$ molecules as well as MMP-1, MMP-3 and TIMP-1 at the synovial cartilage junction.

Adhesion molecules such as the $\beta 1$ integrins are critically important for the binding of inflammatory cells to ECM components, such as fibronectin and collagen^{9,10)}. The present study describes the in situ staining patterns of the $\alpha 4$ and $\alpha 5$ members of the $\beta 1$ integrin family of the cells bordering the interface between the pannus and the cartilage surface. $\alpha 4 \beta 1$ and $\alpha 5 \beta 1$ positive cells were present in large numbers in the pannus, the $\alpha 5 \beta 1$ positive cells usually outnumbering the $\alpha 4 \beta 1$ positive cells. The increased cartilage-pannus junction staining for $\alpha 4 \beta 1$ and $\alpha 5 \beta 1$, associated with binding to cartilage matrix, may result in the activation of the pannus cells. Since these integrins function as fibronectin receptors²⁹⁾, it seems likely that the fibronectin-rich environment of the rheumatoid cartilage surface³⁰⁾ may effectively bind pannus cells expressing high levels of these molecules.

The strong expression of $\alpha 5 \beta 1$ on chondrocytes, as observed in the present study, would suggest that interactions between chondrocytes and fibronectin may occur in the course of pannus formation through the activation of chondrocytes by various cytokines^{7,31)}. Many of the known ligands for integrins, including collagen, fibronectin, laminin and thrombospondin, are present in articular cartilage. The role of integrins in collagen-chondrocyte interaction is, as yet unclear^{32,33)}. Recent studies suggest that certain chondrocyte-ECM interactions may be mediated by integrins^{29,31,34,35)}.

It is unknown whether the invasive

behavior of inflammatory cells may be dependent on the action of proteases, as has been observed in the metastasis of solid tumors¹⁶⁾. Recently, it has been shown that the expression of the fibronectin-degrading protease is down-regulated by crosslinking of $\alpha 4 \beta 1$ integrin receptors on T lymphocytes¹⁵⁾. It was concluded that the expression of these enzymes is controlled partly by lymphocyte activation signals and by direct signalling via $\beta 1$ integrins. The cells that infiltrate the pannus are made up mainly of fibroblasts, macrophages and lymphocytes. All these cells produce and secrete matrix metalloproteinases with cell-specific patterns of the individual MMPs. These appear to be prerequisites for the induction and control of expression of MMPs and their functional roles in the mediation of immunity and inflammation³⁶⁾.

The molecular mechanisms responsible for the invasiveness and collagenolytic properties of the pannus are not clear. Fibroblast-type collagenase or MMP-1 has long been considered as the key enzyme in the destruction of collagen at the pannus-cartilage junction^{37,38)}.

In the present study, MMP-1 and MMP-3 are strongly expressed on synovial lining cells and cells penetrating cartilage. Furthermore, these components of the pannus were strongly stained in chondrocytes beneath the pannus and in pericellular lacunae. These observations strongly suggest that matrix metalloproteinases such as MMP-1 and MMP-3 digest the cartilage

matrix. MMP-1 cleaves interstitial types I, II and III collagen, and MMP-3 exhibits a broader substrate specificity, hydrolyzing collagens found in basement membrane, as well as glycoproteins (fibronectin and laminin) and proteoglycans³⁶⁾. It has recently been shown that in contrast to MMP-1, MMP-3 is very active against cartilage-type II collagen and aggrecans^{39,40)}. These investigation concluded that two enzymes, an interstitial collagenase and gelatinase, are required for the complete dissolution of stromal collagen during cellular invasion.

In the present study, TIMP-1 was weakly stained at the cartilage-pannus junction. Synovial lining cells also showed very weak staining. The regulation of MMP activity is complex and closely controlled by TIMPs. Specific tissue inhibitors of TIMP provide fine control by binding to this component in a 1:1 complex with MMPs and inhibiting their action⁴¹⁾. The reason for the low levels of TIMP-1 secretion by pannus tissue or RA synovium is unclear. As suggested by Jackson et al.⁴²⁾ it is possible that reduced TIMP-1 secretion by rheumatoid synovium is an acquired functional abnormality of infiltrated cells. Also it has been suggested that excessive MMP activity is a major causative factor in joint destruction in RA^{43,44)}. It appears likely to us that the increased MMP activity of the RA synovium also contributes to joint destruction by promoting angiogenesis. Normal articular cartilage is avascular and resistant to invasion by new vessel growth. In RA, this barrier

is broken and blood vessels grow into the articular cartilage as a result of β 1 integrin expression, elevated proteolytic activity of the MMPs and decreased levels of TIMP-1, and in this way contributing to cartilage destruction.

Acknowledgements

The authors wish to thank Dr. Morris Ziff, PhD., M.D., Ashbel Smith professor Emeritus of Internal Medicine, The University of Texas, Southwestern Medical Center at Dallas, for help with this manuscript.

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