Chapter 2
Molecular Scissors: From Biomaterials Implant to Tissue Remodeling

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2.1 Introduction

As long as healthcare system improves and directs itself toward tissue substitution or replacement, all special materials with biomedical applications will become milestones for clinical and laboratory investigations [30, 56, 114]. We are now living in a dynamic world that is not yet prepared for a "*cyborg*" concept but still dreams about perfect tissue engineering, resulting in organ cloning. Thus, our age belongs to biomaterials and their interaction with living tissues. Tissue-implant interactions are well documented in the last decade, especially those regarding mobile cells in the blood, vascular endothelial cells and cells of various connectives [6]. All type of implantations, especially for non-resorptive materials (metallic, hard insoluble polymers) [99] involve tissue trauma, which induces an inflammatory response, followed by wound healing reaction (angiogenesis, fibroblast activation) and extracellular matrix (ECM) remodeling [65]. At the same time, any material intended for implantation should not impede cell development, growth or multiplication, in close vicinity or at certain distance. Moreover, all high performance implanted materials should limit the extension of inflammatory reactions and also should promote tissue remodeling toward a functional status [112, 113, 116].

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I. Antoniac (ed.), *Biologically Responsive Biomaterials for Tissue Engineering*,
Springer Series in Biomaterials Science and Engineering 1,
DOI 10.1007/978-1-4614-4328-5_2,

Most of the implanted biomaterials, mainly bone prostheses lead to an overstated healing reaction as is fibrosis [112]. ECM production can also induce increased cell productivity. It is well documented, even if still poorly understood, that inflammatory processes may influence good biocompatibility, while tissue remodeling following inflammation plays an essential role in accurate organ function restoration [44, 112]. The ultimate goal for an implantable biomaterial should be the bioactivity, the capacity to support tissue regeneration and few or lack of fibrotic tissue.

An implanted biomaterial interacts not only with mobile cells or physiological body fluids but also with ECM. Almost all tissues (except epithelial tissues) possess an abundant ECM, with various compositions. Many implant failures may be due to an impaired cellular response (including inflammation) but all cell behavior can be influenced by the chemical composition and the physical properties of the ECM [57, 116]. The ECM represents a complex network of proteins and polysaccharides spread between the cells and which may include many types of molecules secreted by cells [15]. More than universal biological glue, ECM also forms highly specialized structures such as cartilage, tendons, basal laminas, and bone and teeth with some forms of calcium phosphate crystals [41, 81, 107]. The quantitative variations of different types of matrix macromolecules determine the diversity of the matrix shapes, consistent with the tissue functions. Thus, in the epithelial tissues the ECM is limited in volume, but in the connective tissues its volume is much larger than the cell's volume.

ECM plays an active and complex role, influencing cell development, migration, and proliferation while stabilizing the shapes and cell metabolic functions. During the differentiation process the cells require specific components. Cells morphogenesis depends closely on the ECM fibers. The ECM components can bind growth factors and hormones offering signal abundance for neighbor cells. While different cells may secrete various elements of ECM, we may distinguish two types [13, 49, 60, 75]:

1. Polysaccharide chains of glycosaminoglycans, with a covalent bound to a protein, forming proteoglycans.
2. Fibrillar proteins, which could be either structural (collagens and elastins) or adhesive (fibronectin and laminin).

Glycosaminoglycans and proteoglycans molecules from connective tissues form a highly hydrated gel-like essential substance in which collagen fibers are embedded. These collagen fibers strengthen up and help to organize the ECM while polysaccharide gel gives resistance to pressure. At the same time, the aqueous phase of the polysaccharide gel allows a fast nutrients, metabolites, and hormone diffusion between blood and tissues. Also, the elastin fibers enhance the ECM-specific elasticity, while some proteins as fibronectin are responsible for fibroblast or other cells' attachment to ECM in the connective tissues. For epithelial cells, laminin connects them to basement membrane (BM) [12, 47].

2.2 Extracellular Matrix Tailoring by Specific Means

Controlled interactions between ECM and mature differentiated cells may induce important changes in cells' behavior following specific signaling pathways [25, 58, 67]. Matrix tailoring supposes not only pure ECM dissection and cleavage for its main molecules but also subtle changes in ECM composition, which may alter the activity of the growth factors or of cells' surface receptors. In order for the essential biological processes to develop, ECM must be degraded in a controlled manner in order to allow cell displacement during embryonic development, tissue remodeling, and tissue repair [1, 85]. However, the same process of ECM degradation, if exceeded by the impairment of natural inhibitors, participates as innocent bystander or by active facilitator to growth, invasion, and metastasis of malignant cells. These processes are directly mediated by Matrix Metalloproteinases (MMPs), which we may consider to act as true molecular scissors for ECM.

2.3 Proteases and MMPs Family

All MMPs are included in the main class of proteases. These proteases include exo- and endopeptidases (also called proteinases). These proteinases can be divided in four classes according to the catalytic site and active center: serin/threonin proteinases, cysteine proteinases, aspartic proteinases, and metalloproteinases [20, 39, 71, 88]. Folding similarities are dividing metalloproteinases in clans while an evolutionary classification divides them in families. In metalloproteinases class we may include 8 clans and 40 families [121]. MMP members are included in the MB clan, characterized by a specific three His residues from which the third is bound to the Zn ion [24]. In this clan we can observe two major families (M12 and M10), each with two subfamilies, astacins and reprolysins for the first family and seralysins and matrixins (MMP) for the second. MMPs are gathered together in this subfamily of zinc and calcium-containing endoproteases that have been traditionally characterized by their collective ability to degrade all components of the ECM [121]. These enzymes are supposed to regulate the homeostasis in various tissues under the direct control of tissue inhibitor of metalloproteinases (TIMPs), which bind to and inhibit the activity of MMPs [19, 43]. Accordingly, an imbalance between MMPs and TIMPs can lead to a variety of pathological states, such as metastasis of cancer [29], or diseases including rheumatoid arthritis and multiple sclerosis [32]. At least 28 members of this enzyme family, which demonstrate significant sequence homology, have been reported [19, 22, 87]. Following functional criteria, they can be classified as collagenases (MMP-1, -8, -13, and -18), gelatinases (MMP-2 and -9), stromelysins (MMP-3, -10, and -11), matrilysins (MMP-7 and -26), enamelysin (MMP-20), metalloelastase (MMP-12), and membrane-type MMPs (MMP-14, -15, -16, -17, -24, and -25) [121]. Part of the newly discovered MMPs (after 1996) remain in a separate group, called "others" (MMP-19, -21, -23, -27, -28) while classification undergoes

permanent changes, following new discoveries regarding their functionality [45, 83, 119]. For more than 30 years MMPs have been seen as promising targets for the treatment of the above-mentioned diseases because collagenase (MMP-1), gelatinases (MMP-2 and -9), and stromelysin-I (MMP-3) have been shown to play a key role in cancer invasion and metastasis [8, 19, 23, 82]. A large number of succinyl hydroxamates and sulfonamide hydroxamates have been reported as MMP inhibitors [55, 62, 106, 125]. Accordingly inhibitors of the gelatinases may have therapeutic potential for angiogenesis and/or tumor metastasis [28, 70, 90, 110].

Some authors [8, 16, 88] have nominated these enzymes as metzyncins due to the fact that they contain a Met residue in a carboxylic area near catalytic Zn ion. In this area, the polypeptidic chain of the catalytic site is bending and this bend influences the catalytic pocket activity. All and each detail regarding the catalytic site is important regarding the action of natural and synthetic inhibitors that are able to modulate MMP activity, regarding ECM degradation or tissue remodeling. In 1995, Krane [68] has formulated the principles for an MMP to be considered as an active factor for remodeling processes:

- Remodeling may be blocked by a drug or specific antibody directed against that specific MMP.
- Remodeling process may be reproduced by MMP over-expression on transgenic animals.
- Remodeling process may be interfered by specific MMP gene deletion.
- Spontaneous mutations can be identified and phenotypic characterization realized.
- Mutations can be induced in genes that reproduce the remodeling processes.

Thus, modulation of MMP activity appears to become essential in remodeling control, even in implant adaptation to host.

The far most important domain of the MMPs is the catalytic one [93, 120]. However we cannot ignore the other domains of this enzyme that include two Zn ions (one catalytic and one structural) and 2–3 Ca ions (Fig. 2.1). Starting from the N-terminus, we distinguish the following: (1) the signal peptide (12–20 hydrophonic amino acid residues). MMP-17 is lacking this domain; (2) pro-peptide includes around 80 residues and a highly conserved region toward the C-terminus; this end includes the Cys residue found in all MMPs, responsible for catalytic Zn contact and the stability for the zymogenic (inactive) form of the enzyme; (3) furin-cleavage domain includes around nine residues leading to furin cleavage; Only MMP 11, 14, 15, 16, and 17 include this domain while the other MMPs are cleaved by extracellular proteases, exposing the Zn ion and inducing autolytic cleavage for the remaining peptide; (4) catalytic domain, including approximately 160–170 residues, contains the Zn and Ca ions; (5) the 54-amino acid C-terminal region contains the catalytic Zn; (6) three repetitive fibronectin-like domains (mainly in MMP-2 and -9, serving for gelatin binding); (7) flexion region connects the catalytic domain to the next hemopexinic domain; (8) hemopexinic domain, including around 200 residues with four repeats that simulate hemopexin and vitronectin; this domain is involved in the recognition of the specific natural inhibitors (TIMPs); (9) membrane insertion domain, mainly for membrane type matrix metalloproteinases (MT-MMPs) with a length of 80–100 residues.

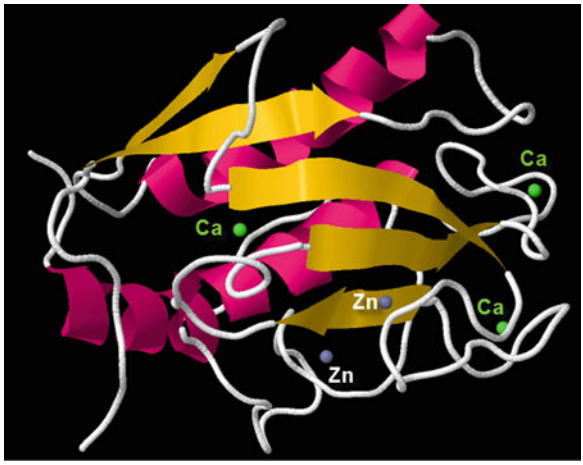

Fig. 2.1 Gelatinase A (MMP-2) catalytic domain, indicating the Zn ions coordinated by three histidines each and three Ca ions

2.4 MMPs' Activity Depends on Catalytic Domain Structure and Behavior

Modulation of MMP activity depends directly on the specific inhibition or modulation of the catalytic domain. This inhibition depends on the intimate structure of the catalytic domain of the MMPs (which is common to almost all metalloproteinases) [93].

The catalytic domain of the MMPs exhibit the shape of an oblate ellipsoid. In the "standard" orientation, as depicted in most papers regarding MMPs [10, 11, 54] as preferred for this enzyme display, a small active-site cleft notched into the ellipsoid surface extends horizontally across the domain to bind peptide substrates from left to right. The MMP active site is composed of two separate regions: a groove at the protein surface surrounding the protruded catalytic Zn ion and a specificity site, nominated as S1′; the latter shows a higher degree of variability among the MMP in the family. Inside the groove, all tested synthetic inhibitors adopt extended conformations, express hydrogen bonds with the enzyme, and provide support for Zn ion coordination (see Fig. 2.1). Most current pharmacologic interventions have focused on development of MMP synthetic inhibitors that target MMPs in the extracellular space. MMP synthetic inhibitors are included into four categories: peptidomimetics, nonpeptidomimetics, tetracycline derivatives, and bisphophonates [55, 105]. Peptidomimetic inhibitors mimic the structure of collagen (a common substrate of MMPs) at the MMP cleavage site [51]. These compounds function as competitive inhibitors and chelate the catalytic zinc atom. Depending on the group that binds and chelates the zinc atom, peptidomimetic inhibitors for MMPs can be further subdivided into hydroxyamates, carboxylates, aminocarboxylates, sulfhydryls, and phosphoric acid derivatives. The hydroxamate MMPIs (batimastat, BB-1101 and marimastat) are broad spectrum inhibitors with significant activity in several animal models. Batimastat was the first MMPI to enter clinical trials but its utility was limited due to poor water solubility which required intraperitoneal administration

[69, 94]. Marimastat is a broad spectrum synthetic MMP inhibitor [63, 108] acting on MMP-1, -2, -3, -7 and -9—with good oral bioavailability that was studied in phase II and III clinical trials in many tumor types [40, 117].

The collection of universal chemical properties that characterizes the specific action of a ligand in the active site of a three-dimensional conformational model of a molecule is called a pharmacophore. The properties of the active catalytic site in an MMP are figured by the presence of hydrogen bridges, electrostatic interactions with an alleged inhibitor or hydrophobic areas. Pharmacophore modeling by computational processing becomes a universal, comprehensive and editable process. The inhibitor-catalytic site selectivity can be adjusted by adding or omitting some characteristics. Our group has used the LigandScout 2.0 software [123, 124] in order to align pharmacophores with important ligand molecules, based on their properties, in arbitrary combinations. The alignment of the two elements is realized by pairing only, regardless of the number of aligned elements. Thus, it is required to define a structure as marker element. In order to define the pharmacophores, from the 21 MMP-inhibitor complexes we have chosen nine files that are crystallographically defined in PDB databank: 1eub, 1fls, 1xuc, 1xud, 1xur, 1you, 1ztq, 456c, and 830c, respectively. We have chosen these complexes with experimentally defined affinity constants for a further usage of the pharmacophores, based on these determined complexes. The pharmacophores represent important filters regarding in silico evaluation of new MMP inhibitors. For automatic generation of MMP pharmacophore models we have imported the selected PDB files in LigandScout application. Due to the fact that PDB files do not include information regarding atom hybridization status and bond type, LigandScout application uses a complex algorithm to analyze the ligand structure in order to assign the bond type according to molecular geometry. Thus, it is recommended a manual check for the automatic deduction for the ligand structure. The key structure for the inhibitors CGS 27023 and WAY-151693 in the complex structure 1eub and 1fls respectively in PDB database is the isopropyl substituent and the basic 3-pyridyl substituent. Zn coordination is insured by the hydroxamic group while this inhibitor is part of the sulfonamide hydroxamates inhibitor family [48]. The non-hydroxamic inhibitor WAY-170523 in the complex 1ztq shows the replacement of the hydroxamic acid group with a carboxylate group; the latter is not Zn chelating but the inhibitory action is due to correct positioning of the benzofuran moiety of the P1′ group by a biphenyl P1′ linker that fills the hydrophobic S1′ tunnel [94].

Another inhibitor, found in the 1xuc, 1xud, and 1xur complex is represented by pyrimidine dicarboxamide [17]. This inhibitor binds deeply in the S1′ pocket and the pyridyl substitutions do not approach the catalytic zinc more than 5.5 Å. This inhibitor shows a bent conformation with the pyridyl substitutions close to Leu218.

For the pyrimidinetrione-based inhibitors of MMP13 in 1you PDB file, the aryloxyaryl ether fits in the S1 pocket while the pyrimidinetrione binds to the active zinc in the catalytic site [4, 14, 33, 97]. The three dimensional alignment of two inhibitors shown a score of around 77 from 100, that is remarkable for two different inhibitors in two different crystallographic determined structures.

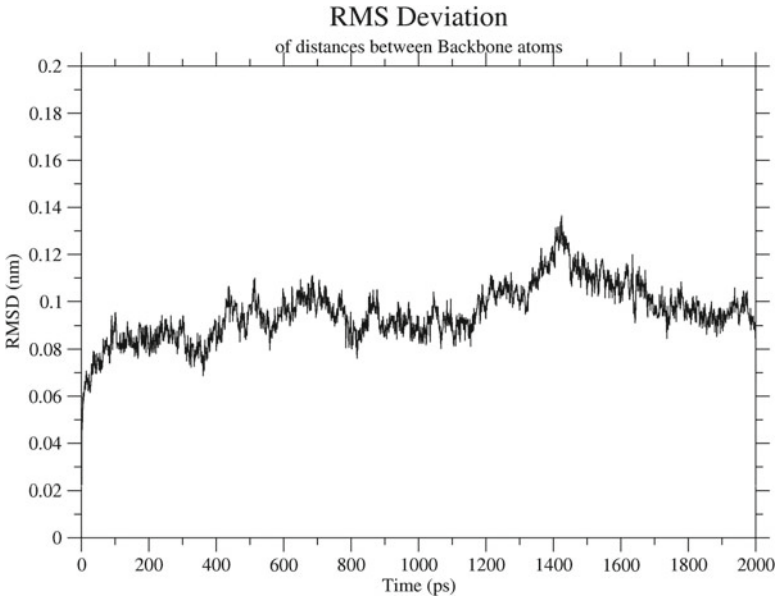

Fig. 2.2 Root mean square displacement (RMSD) for backbone atoms on the 1QIB file, representing the catalytic site of MMP-2, during a 2 ns molecular dynamic simulation

It is demonstrated that various MMPs exhibit different selectivity for many ECM proteins. Thus, it is important to understand such substrate selectivity to develop new synthetic MMP inhibitors. We have also shown that not only the Zn ion coordination in the P site is important, but also the hydrophobicity of S1 tunnel can be a step in further computer design for potent inhibitor or enzyme modulation factors.

The stability of the catalytic domain was subject for many studies [4, 26, 27] including our group investigation [92]. In order to appreciate this stability, we have performed molecular dynamics (MD) simulations of the catalytic domain of a known matrix metalloproteinase (MMP-2) for a 2 ns duration, in the absence of the substrate or a known inhibitor, starting from Protein Data Bank published data (ID-1QIB) [9]. We have chosen to the MMP2 (gelatinase A) catalytic domain, while it is the best characterized crystallographically. We have evaluated the conformational stability for MMP2 catalytic site during MD simulation.

In order to describe the variation of atom position into the simulated structures by comparison with the experimental defined conformation, it is necessary to plot the Root Mean Square Displacement (RMSD) over time (Fig. 2.2). We have tried to assess the stable states of the investigated by monitoring the RMSD of the protein backbone or only for the C-α carbon atoms along the trajectory, compared to the structure before starting the simulation. Literature data [18, 26, 100] show that RMSD values ranged between 0.1 nm and 0.3 nm, if constant, are representing a

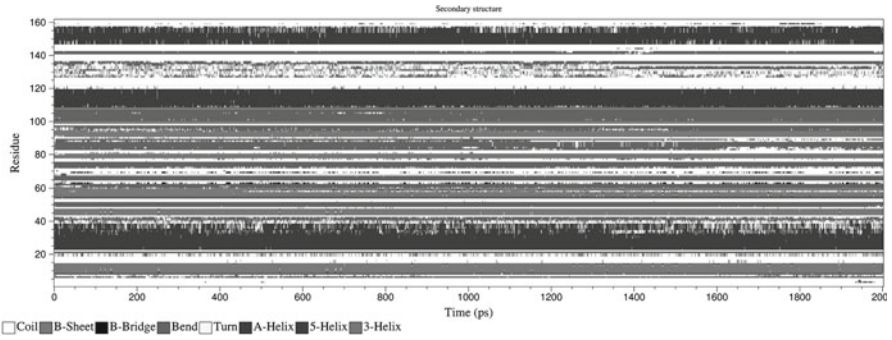

Fig. 2.3 Conservation of the secondary structure of the MMP2 catalytic site during the 2 ns MD simulation

conformational stability status for the simulated molecule. We have isolated the obtained trajectories for the catalytic domain of MMP-2 in the 1QIB file and we have analyzed the evolution of the secondary structure within simulation time. The domains of the catalytic site showed a good conservation over simulation time (Fig. 2.3).

By performing a Root Mean Square Fluctuation (RMSF) analysis, we have compared the NMR starting structure of the MMP2 catalytic site with the structures obtained by molecular dynamics simulations and we have noticed a high stability for this domain. The catalytic site stability was witnessed by the reduced fluctuations for the catalytic Zn area. However we have noticed some fluctuations, mainly in some loop regions near the catalytic site of the MMP, in 192–250 region of the MMP2 PDB file (394–449 on drawing) (Fig. 2.4).

Protein conformational changes are important mainly for enzymes and their activation status. These conformational changes are part of the kinetic states for proteins and include the variability of the secondary and tertiary structure and thus the global atomic movement in the investigated protein-enzyme. At the same time, side chain fluctuations and loop movements are characterizing the kinetic states of the observed substates in molecular dynamics simulations. MMP-2 shows a remarkable stability for the considered catalytic site and the presence of two substates. The analysis of free Gibbs energy landscape for MMP2 catalytic site showed a native state with two substates separated by less than 1KT (Fig. 2.5).

Thus, we have concluded that the dynamic signature for the MMP2 catalytic site is a native state with two substates, in close vicinity. We have also studied the secondary structure conservation, root mean square deviation (RMSD), and fluctuation cluster analysis for a full structured description of the crystal structure of the MMP-2 catalytic site. Based on these results, we can thus define new tricks for strategies regarding MMP inhibitors design. The role of the atomic position in the catalytic site for MMPs appear to be essential regarding to further simulations for conceiving a rather modulating inhibitor for these enzymes.

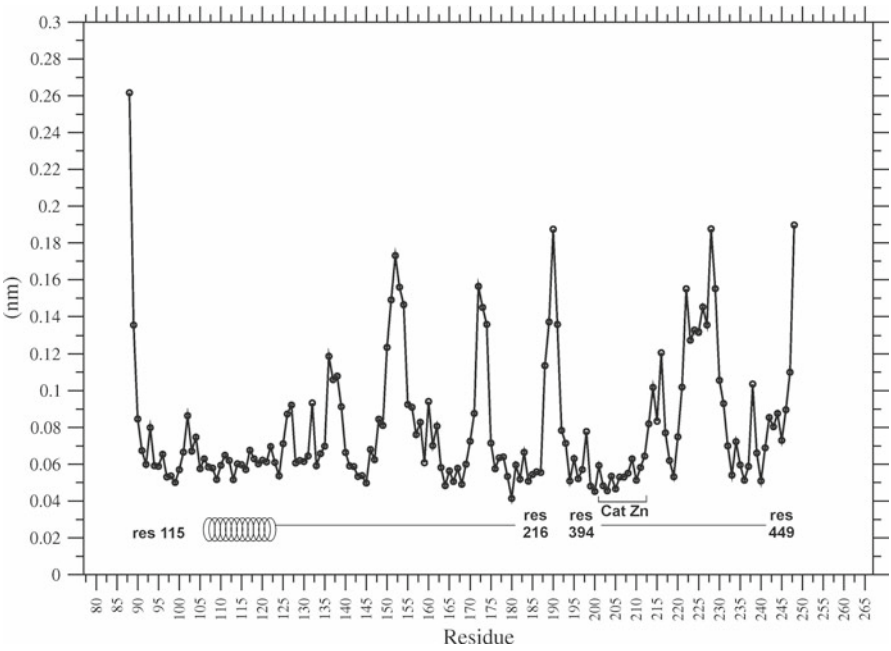

Fig. 2.4 Root mean square fluctuation (RMSF) for MMP2 catalytic site in 1QIB file

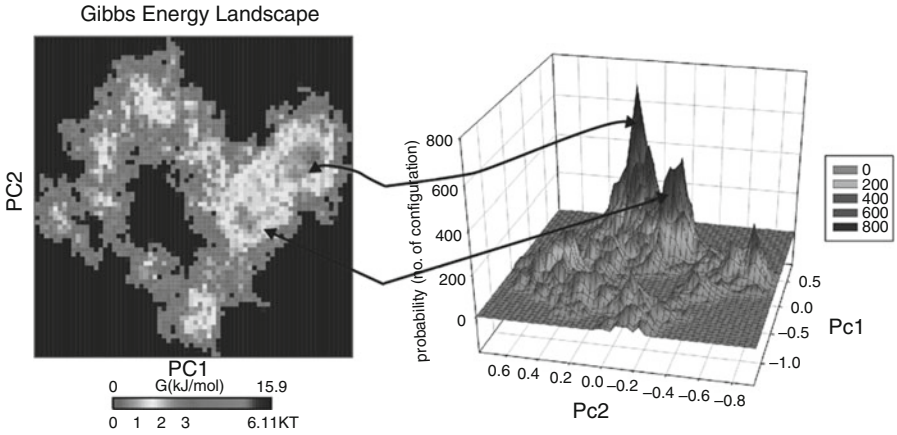

Fig. 2.5 Free Gibbs energy landscape for MMP2 catalytic site with two substates separated by less than 1KT

2.5 MMPs Natural Inhibitors

All MMPs are subject to natural inhibition by specific proteins called tissue inhibitors of matrix metalloproteinases (TIMPs). TIMPs bind to MMP in 1:1 or 2:2 ratios. TIMP-1 represents a more efficient inhibitor for MMP-1, MMP-3, and MMP-9 than

TIMP-2 [38, 79, 127]. Some authors [127] observed that TIMP-2 has a double effect on MMP-2 since MT-MMP-mediated proMMP-2 activation requires a tiny amount of TIMP-2 to make activation progress, whereas a higher concentration of TIMP-2 inhibits MMP-2 [64]. TIMP-3 may inhibit MMP-2 and MMP-9 [122], and TIMP-4 is a good inhibitor for all MMPs [109]. Tissue levels for TIMPs in ECM are largely exceeding MMP levels in the same areas. TIMPs act also as growth hormone and inductor for apoptosis (mainly TIMP3). TIMPs transcription process is regulated by cytokines and growth factors, involved in inflammation and remodeling processes. Natural inhibitors for MMP may represent important performers in this play. Some authors have described the close relation between MMP-2-TIMP-2 and MMP-9-TIMP-1 enzyme-inhibitor pairs [86]. Interactions between these pairs are indeed very complex and widely investigated from various points of view, by different experimental means [102] and for different purposes. TIMP-2 is involved in MMP-2 activation at the cell surface by forming a complex with MT1-MMP. Thus, TIMP-2 can play a dual role during interaction with MMP-2. The biochemical relationship between TIMP-2 and MMP-2 may induce surface-mediated enzyme activation, while simple interaction TIMP-2–MMP-2 can determine enzyme inhibition. Our group has also investigated the colocalization and complex relationship between MMP-2, MMP-9 and TIMP-2, by molecular docking studies [92] to evaluate the docking options for these enzymes and inhibitor. While we have found described in the literature only the interaction MMP-9–TIMP-1, we have investigated the possible interaction between MMP-9 and TIMP-2 by molecular docking means. Thus we have evaluated first the optimal positions of the TIMP-2 around MMP-2 molecule and then, using a similitude procedure, we did the same thing for MMP-9–TIMP-2 complexes, for which there were no data regarding direct interaction. The investigated docking molecular models were MMP2–TIMP2 and MMP-9–TIMP-2. For the MMP-2–TIMP-2 enzyme-natural inhibitor complex, we have selected the 1gxd file from Protein Data Bank (PDB) [9] from which there were picked individually the molecules involved in this interaction, MMP-2 and TIMP-2. This selection was followed by an identification of the specific regions involved in the enzyme-inhibitor interactions, mainly the catalytic site and the hemopexinic site for MMP-2. This enzyme has the unique property of being able to bind successively two TIMP-2 molecules, either on hemopexinic site (playing a role in enzyme activation) or on the catalytic site (playing a role in enzyme inhibition). The docking process starts from two known extracted structures that form a complex (MMP2–TIMP2 in 1gxd file); the extraction of these proteins from the complex should not alter the initial structure of the complex; this procedure was then validated by superposition. We have checked this algorithm for this modeling software, while there is no available data regarding the interaction MMP-9–TIMP-2. Following rigid docking and filtering, we have a picture of the most probable positions for the inhibitor around the enzyme. The filtering procedure was performed for localizing the catalytic and the hemopexinic sites for TIMP-2 binding. The two resulted structures for MMP-2 and TIMP-2 (that occupies the best position near the catalytic site) were superposed over the crystallographic model represented in the 1gxd file from PDB database. Following superposition we have calculated also the Root Mean Square deviation

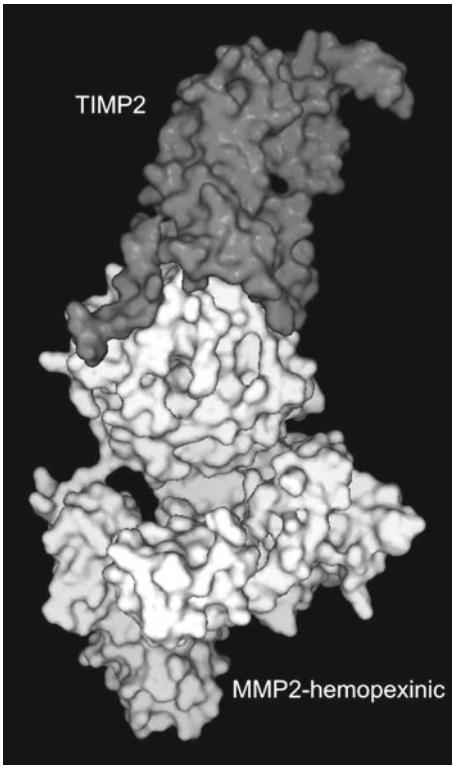

Fig. 2.6 Final refined complex final MMP2 (hemopexinic)—TIMP2, with the choice of the position with the most favorable score for docking

(RMSd), the distance between the initial atom coordinates in the crystallographic structure and the final coordinates of the partially altered structures by the modeling process. RMSd values of 2.30 Å indicated a correct superimposition and validated the method for further enzyme inhibitor pairs. After model refinement, we have represented the results with VMD viewer [53]. We have represented the final complexes MMP-2 (catalytic-refined)–TIMP-2 (Fig. 2.6) and MMP2 (hemopexinic-refined)–TIMP2 (Fig. 2.7).

MMP-9–TIMP-2 model raised some problems while PDB databases do not include yet a crystallographic model but only a theoretical one. The chosen model was 1LKG. In PDB database we have selected the hemopexinic domains (1ITV-dimeric form) and catalytic (1L6J) [9]. The 1L6J file contains the whole MMP9 sequence from which we have selected the catalytic domain for the experimental model. The domains were superimposed over the theoretical model for MMP-9 (Fig. 2.8).

The docking procedure for the MMP9–TIMP2 complex determined 10,000 possible variants that have been filtered and selected from the minimal coupling energy then graphically represented. Following this molecular docking study we have concluded that the natural inhibitor TIMP2 has a higher affinity for the catalytic

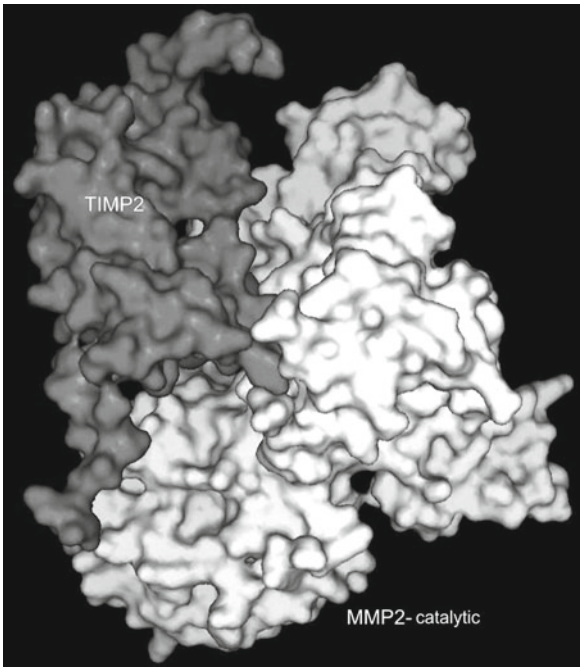

Fig. 2.7 Final refined complex MMP2 (catalytic)—TIMP2, with the choice of the position with the most favorable score for docking

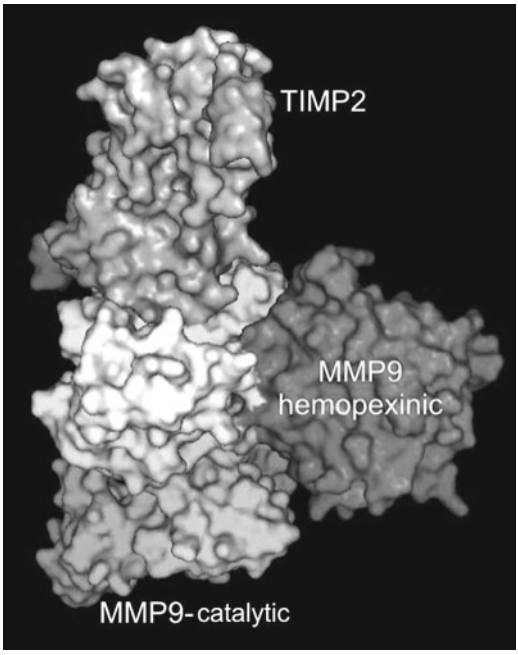

Fig. 2.8 Alignment for hemopexinic and catalytic domains with the theoretical model of MMP9

domain than for the hemopexinic domain of MMP. Regarding the activation mechanism, these data correspond to the well-known experimental results [7, 31, 77], that sustain that initially; TIMP2 forms an activation complex with the hemopexinic domain in MMP2.

Even if has a higher affinity for TIMP2, the catalytic domain cannot bind it while the specific binding site is inactive in the pro-form of the enzyme. Following MMP2 activation and removal of the pro-domain of the enzyme, the catalytic site becomes available and the favorable free binding energy will induce either the substrate binding or the natural inhibitor TIMP2. We have confirmed that MMP2 can consecutively bind two TIMP2 molecules, one for the activation complex at the hemopexinic domain level and the other at the catalytic site, inducing inhibition. For the MMP9–TIMP2 complex, the affinity for the hemopexinic and the catalytic site was almost the same. Thus, the activation-inhibition model in this triad could be [91]: MMP2 together with TIMP2 and MT-MMP1 forms an activation complex that activates MMP2; the activated MMP2 attacks the specific substrate. At the same time TIMP2 will inhibit MMP9 at the catalytic site while the hemopexinic site is already coupled with TIMP1 that participates to its activation.

2.6 MMPs in Wound Repair

MMPs participate to all wound repair steps following biomaterial implantation (Fig. 2.9) [34, 73].

Inflammation step is mediated by cytokines and chemokines incoming from resident cells in the tissue to which implantation is addressed (epithelial or endothelial cells, fibroblasts). MMPs can activate the inflammatory mediators at cell surface either by direct processing (cleavage of a pro-domain) or even by degradation, thus inhibiting pro-inflammatory signals. MMPs are also able to cleave molecular domains of the intercellular or cell-matrix junctions in epithelia in order to promote re-epithelization. Moreover, MMPs are involved in scar tissue remodeling either by direct proteolytic cleavage of collagen molecules or indirect, by influencing cell behavior. ECM processing is complete at the end of wound repair process but is also involved in regulation of the inflammatory process. MMPs are playing key regulation factor roles regarding multiple processes in tissue repair [52, 98].

MMPs are also playing important roles in inflammatory processes. As we have already emphasized, inflammation is one of the first processes at the biomaterial implant site. Inflammation processes are associated to leukocyte influx and activation and are influenced by antimicrobial peptides, lipid mediators, receptors, chemokines, and cytokines, fragments derived from ECM. Production and activation of these factors is controlled by effectors that include MMPs. Specific MMPs derived from epithelial cells are regulating numerous steps during inflammation, as leukocyte trans-epithelial migration, activity, and distribution of chemokines [21]. Inflammatory cells are expressing MMPs as all other epithelial

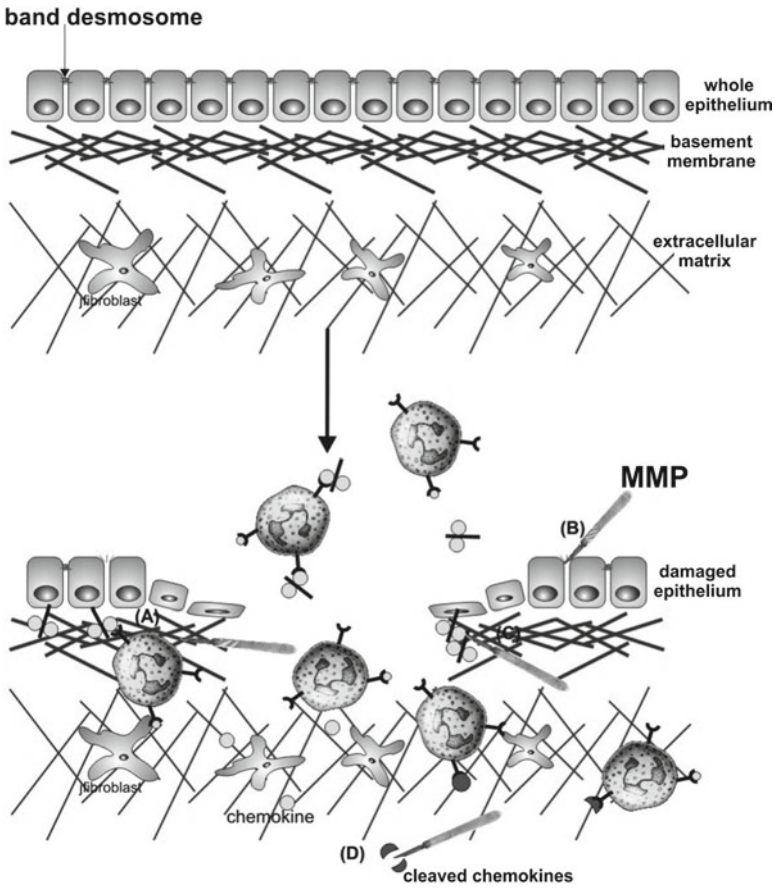

Fig. 2.9 MMPs involvement in healing steps. (**a**) Cell migration is facilitated by MMPs by degradation of the adhesive and ECM proteins; (**b**) Re-epithelization—adhesion proteins are cleaved by MMPs and cells involved in healing are promoted; (**c**) Leukocyte influx—chemokines and proteoglycans are cleaved by MMPs; (**d**) Inflammation step; MMPs are processing multiple chemokines

and stromal cells in wounds. In wound repair processes we may observe the involvement of MMP-1, 2, 3, 7, 9, 10, or 28. Many of these MMPs may regulate chemokine activity by direct proteolysis or by generating chemokine gradients. Chemokines are divided in subfamilies based on N-terminal cysteinic residues and play specific roles in attracting the leukocyte flow toward inflammation site. CC chemokines are characterized by two neighbor cysteinic residues toward N-terminus and are responsible for monocyte chemotaxis. CXC chemokines show another amino acid between cysteine residues and are responsible for neutrophil attraction toward inflammation site. Chemokine effects are mediated by binding to 7-domain-transmembrane G protein-coupled receptors (7-TM-GPCRs). MMP-controlled chemokine cleavage induces a reduction of chemokine activity.

This mechanism characterizes CC-chemokines that are producing antagonists for the receptors that will inhibit signaling pathways, following MMP processing [126]. It appears that chemokines CCL-2, 5, 7, 8, and 13 are more efficiently and frequently activated by MMP-1 and -3. CXC chemokines show variable response following cleavage by MMPs. Some chemokines are resistant to MMP processing (CXCL1, -2 and -3) while others are processed by multiple MMPs (CXCL5, -12 and interleukin-8). Human CXCL8 [35] is processed by MMP-8 and -9 and induce their activation. CXCL5 (LIX) chemokine [66] is processed by MMP 1, 2, 8, 9, and 13. This chemokine processing determines an increase of its activity that promotes an accelerated recruitment of the inflammatory cells. CXCL12 (SDF1) chemokine is also processed by multiple MMPs (1, 2, 3, 9, 13, 14) and its processing induces a decrease of the chemokine activity [42]. From all MMPs, it appears that MMP-1, -3, and -9 are the most potent agents for modulating chemokine signaling activity.

Other MMPs (disintegrins) are able to cleave CXCL16 at cell surface, allowing binding to specific receptor and regulating the T-cell activity in wound repair [2, 36, 111]. MMPs are also required to define chemotactic gradient. MMP-7 represents a key factor for regulating transepithelial neutrophils migration. Injured epithelial cells are expressing CXCL1 that bind to heparan-sulfate glycosaminoglycan chains and to syndecan-1, thus generating a chemical gradient required for activated neutrophils. In the absence of MMP-7, neutrophils are not migrating anymore and are sequestrated into intercellular space. MMPs and disintegrins ADAM's (*A Disintegrin And Metalloproteinase*) play an important role in diapedesis [61]. ADAM-28 is expressed by lymphocytes in the peripheral blood and is located as membrane coupled secreted enzymes [50]. It may bind the selectin glycoprotein at lymphocyte surface thus activating P-selectin binding on endothelial cells. MMPs are able to cleave occludins from tight junctions and VE-cadherins (vascular endothelial) from adhesion junctions, being involved in endothelial permeability [96]. Inflammation represents a main step in wound healing and in tissue early response to implant. Peri-implant reactivity following biomaterial implantation into soft tissues involves the follow-up for inflammatory reactions, necrosis, granuloma presence dystrophy or calcification reactions around implanted tissue [5]. These reactions are induced and depend on the germ contamination of the implanted materials. Inflammatory reaction is notable from 2 to 4 weeks, being usually moderate and limited to a very thin area around the implant, regardless the biomaterial physical properties. Between 4 and 12 weeks the fibrous capsule shows two separate areas: a layer of inflammatory cells with a moderate inflammatory response more reduced than the previous interval and a fibrous area formed by a collagen network and fibroblasts. After 26 weeks, only a thin fibrous capsule remains, where macrophages and inflammatory cells are missing. We can describe new formed capillaries between the fibrous capsule and the surrounding tissues [72].

Our group has performed various biomaterials testing in vivo by subcutaneous rat implantation followed by analysis of peri-implant tissues in order to detect inflammatory processes and their consequences. Following some collagenated

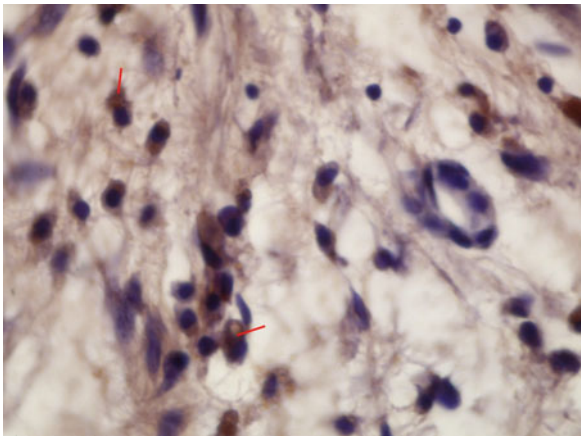

Fig. 2.10 MMP8 positive lymphocytes, detected by immunohistochemistry, around small capillaries

networked biomaterial implant, at 16 weeks following implantation; we have observed a specific periimplant fine fibrous capsule [84]. In this capsule we have detected, by immunohistochemical means, the presence of MMP-8 in few lymphocytes, close to fine capillaries (Fig. 2.10).

A specific role is played by MMPs during proliferative step of the wound repair process [34]. Beside proliferation and epithelization induced by macrophages that are stimulating the fibroblasts using interleukin-1 (IL-1) and keratinocyte growth factor -2 (KGF-2), the ECM matrix synthesis is augmented by the same macrophages. They are initiating the formation of platelet-derived growth factor (PDGF) and tumor necrosis factor α (TNF-α). Macrophages are also delivering proteases that are activating the transforming growth factor β (TGF-β). The later stimulates collagen production by fibroblasts. It also prevents collagen degradation by stimulating TIMP secretion, induces fibronectin secretion and increases the expression of integrin receptors to allow cell adhesion to ECM [37, 89].

2.7 MMPs in Epithelial Repair

A main process in wound repair is represented by re-epithelization in which epithelial cells are regenerated on a denudated surface. Re-epithelization supposes the loss of intercellular and cell-matrix contacts near the wound edges while cells are starting to migrate toward the lesion. May MMPs are involved in this wound repair step, mainly MMP-1, -3, -7, 9, -10, -14 and -28 [3, 74, 89].

MMP-1 (collagenase-1) is found in cutaneous wounds in re-epithelization step and its expression ceases while wound repair is complete. In the wounded tegument, keratinocyte migration from the basement membrane meets a rich-collagen dermal matrix, and binding to this element of the ECM (which is intermediated by α2β1 integrin) stimulates MMP-1 expression and also for other MMP genes. MMP-1

facilitates keratinocyte migration in dermal matrix by weakening the interactions between collagen and integrins [46]. MMP-10 (stromelysin-2) is colocalized with MMP-1 while MMP-3 (stromelysin-1) is localized in the cells behind the migration wave. Lack of superposition between MMP-3 and -10 (even if they are MMPs with similar structure) suggests that MMPs are playing various roles in re-epithelization processes [103]. MMP-7 regulates re-epithelization by cleavage of E-cadherins in adherence junctions, thus facilitating cell migration. MMP-28 (epilysin) is expressed by keratinocytes localized distally from injured area and lacks from migrating keratinocytes. This suggests a possible role in reorganization of the basement membrane or in cleavage of the adhesion molecules, releasing new cells for the migration wave [103]. MMP-9 (gelatinase B) is also involved in re-epithelization process following local injury [76, 78, 95]. Epidermal growth factor (EGF) and hepatocyte growth factor (HGF) stimulate the keratinocyte migrating ability. Cell migration depends on the MMP-9 activation and it is modulated by TIMPs or by synthetic specific inhibitors. MMP-10 is expressed by epithelial cells in the migrating cell wave and its over-expression may induce aberrant cell migration at wound edge level, reducing the abundance of laminin-5 mainly by direct cleavage. Laminin-5 decreased levels are associated with impairment of the cell-matrix signaling pathways and increase the number of keratinocytes that undergo apoptosis. Proliferation of the epithelial cells behind cell migration wave in the injured area is regulated by MMP-14. It appears that this MMP activity is directly related to keratinocyte growth factor receptors processing during epithelial injury repair. Thus, wound healing is similar to tissue morphogenesis processes that include cell migration, proliferation, differentiation, and cell death [59, 104].

2.8 MMP Involvement in Resolution Step of Wound Healing

It appears that MMP-3 may influence reorganization of actin filaments in dermal cultured fibroblasts that initiates early injured area contraction. It appears that ECM remodeling processes depend on various MMPs as MMP-1, -3, -13, and -14, enzymes that are able to cleave collagen. Also, MMP-7 may process syndecan or elastin. Collagen remodeling that includes degradation of preexistent fibrils and synthesis of new ones represent the main process in wound resolution. The enzymes involved in this process appear to be MMP-2 and -9 together with MMP-3 that are localized at epithelial–stromal interface, behind the epithelial cell migration wave, that suggests involvement in stromal and basement membrane remodeling [85].

2.9 TIMPs in Wound Repair

Inflammatory response is mediated by multiple cytokines and chemokines. Among cytokines involved in acute inflammation, TNF-alpha is activated by cleavage at membrane level by the specific conversion enzyme. One effect on inflammatory

cells (as monocytes) is to stimulate MMP-9 expression by NFkB activation and MAP-kinase signaling pathway. MMP-9 over-expression may impair normal healing and cicatrization process [80, 104, 115, 118].

TIMP-3 is one of the primary inhibitors for ADAM-17 and is involved in inflammation regulation. While TIMP-3 is missing or secreted at low concentrations, ADAM-17 activity is accentuated, inducing TNF alpha secretion and an increase of the inflammatory fluid volume. Interaction between TIMP-3 and ADAM-17 plays an important role in recruiting and redirecting leukocytes. Vascular cell adhesion molecules (VCAM) are also involved in white blood cells recruitment, and during inflammatory response they are cleaved at endothelial cell surface by ADAM-17. TIMP-3 excess blocks soluble VCAM production while the decreases of TIMP-3 levels are activating surface cleavage of VCAM-1 [118].

TIMP-1 may be involved in leukocyte flowing and vascular permeability by regulating apoptotic processes in endothelial cells. TIMP-1 plays an antiapoptotic effect in endothelial cells. It appears that natural inhibitors for MMPs, mainly TIMP-3, are required to adjust inflammatory cell response, by regulating cytokine signaling and cell adhesion receptors.

TIMP also regulates some steps in cell migration. Epithelial cell migration depends on TIMP-1 levels and is impaired by its low concentrations. There are significant differences between epithelial and stromal cell migration during re-epithelization and granulation tissue formation, while TIMP balance these processes [34].

2.10 Evaluation of Immunohistochemical Detection of MMPs in Periimplant Tissues

Our group has performed various tests in order to detect the presence of some MMPs (as gelatinases) in periimplant tissues. We have used simple immunohistochemical detection of MMP-2 and -9, together with the detection of the TIMP-1 and -2, by using a DAB detection system bound to specific primary antibodies against MMPs and TIMPs. For a semiquantitative evaluation of the immunohistochemistry results, we have used the color deconvolution technique, imagined and described by Ruifrok şi Johnston [101]. The analysis of our results showed that MMP-9 is present in various amounts (expressed in area percentage on the examined slides) of 33,010, 27,878, 15,857, 30,213, and 31,852 %, compared to the normal witness slide (without implant) where the MMP-9 marked area was of 9,614 % only. Thus, MMP appear to be involved in ECM remodeling processes in the peri-implant area. TIMP-1 expression is also increased in the peri-implant area, showing that remodeling processes require the presence of the enzyme and its natural inhibitor at the same time.

2.11 Summary

Tissue-implant interactions are well documented in the last decade, especially those regarding mobile cells in the blood, vascular endothelial cells, and cells of various connective tissues. All types of implantation, especially for non-resorptive materials (metallic, hard insoluble polymers) involve tissue trauma, which induces an inflammatory response, followed by wound healing reaction (angiogenesis, fibroblast activation) and ECM remodeling. All high performance implanted materials should limit the extension of inflammatory reactions and also should promote tissue remodeling toward a functional status. Matrix tailoring supposes not only pure ECM dissection and cleavage for its main molecules but also subtle changes in ECM composition, which may alter the activity of the growth factors or of cells' surface receptors. In order for the essential biological processes to develop, ECM must be degraded in a controlled manner in order to allow cell displacement during embryonic development, tissue remodeling, and tissue repair. These processes are directly mediated by Matrix Metalloproteinases (MMPs), that we may consider to act as true molecular scissors for ECM. Modulation of MMP activity appears to become essential in remodeling control, even in implant adaptation to host. Modulation of MMP activity depends directly on the specific inhibition or modulation of the catalytic domain. This inhibition depends on the intimate structure of the catalytic domain of the MMPs (which is common to almost all metalloproteinases). Our group has performed various tests in order to detect the presence of some MMPs (as gelatinases) in periimplant tissues, including molecular modeling studies together with immunohistochemical investigations.

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http://www.springer.com/978-1-4614-4327-8

Biologically Responsive Biomaterials for Tissue Engineering

Antoniac, I.V. (Ed.)
2013, X, 254 p., Hardcover
ISBN: 978-1-4614-4327-8