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Reviews

Human enzymatic activities related to the therapeutic administration of chitin derivatives

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Abstract. Three cases are presented where modified chitins have been extensively administered to volunteers, as dressings for wounded soft and bone tissues, as anticholesterolemic dietary foods, and in the controlled delivery of anti-inflammatory drugs. The interactions of the modified chitins with human enzymes is critically examined. In the context of drug carrier resorption and wound healing, chitooligomers and monomers, generated by lysozyme, *N*-acetylglucosaminidase and human chitinase, activate macrophages and stimulate fibroblasts, respectively; the effects are production of smooth, vascularized and physiologically normal tissues. In the dietary food area, lipase, amylase, 3-hydroxy-3-methylglutaryl CoA reductase, glucokinase and the enzymes of prostaglandin synthesis are involved in the oral administration of chitosan: lipid adsorption is depressed mainly because of the physical form of the chitosan-lipid aggregates, which are unsuitable as substrates. When chitosan is used as a drug carrier, chitosan-drug complexes are present. The uniqueness of chitosan among polysaccharides is underlined in terms of susceptibility to enzymatic depolymerization, cationicity, supply of cell-activating oligomers, and supply of *N*-acetylglucosamine for rebuilding of other biopolymers. Advances in molecular recognition and biocompatibility are also presented.

Key words. Lysozyme; *N*-acetylglucosaminidase, chitinase; 3-hydroxy-3-methylglutaryl CoA reductase; glucokinase; wound dressing; cholesterol; indomethacin.

Introduction

One of the most gratifying aspects of the research carried out on chitin during the last decade is the increased understanding of the beneficial effects of chitin on the human body. Chitin-based tissues are absent in the human body, but *N*-acetylglucosamine, the repeating unit of chitin, and chitobiose are present in glycosaminoglycans and in glycoproteins [1].

In many situations the administration of chitin derivatives to the human body would potentially be helpful in alleviating diseases, preventing sickness or contributing to good health. I shall therefore confine this review to those cases where modified chitins have actually been administered to humans, more precisely in the form of dressings for wounded soft and bone tissues, anticholesterolemic dietary foods and items for the controlled delivery of anti-inflammatory drugs. In these cases, enzymatic activities play essential roles in the biological consequences of the applications of chitosans (Fig. 1).

Other topics of interest, including the antibacterial, antimetastatic, antiarthritic, antiuricemic, antiosteoporotic, immunoadjuvant and other activities, will be found in recent and forthcoming articles [2–11].

The hydrolytic enzymes

Chitinases are classified in families, based on sequence similarities [12, 13]. **Family 18** includes viral, bacterial, fungal, insect, reptile and certain plant chitinases; other members are endo-β-*N*-acetylglucosaminidases which hydrolyse the linkage between the two NAG units in the core of asparagine-linked oligosaccharides in glycoproteins. Eukariotic chitobiases, active on lower chitooligomers, are also present. These enzymes are inhibited by allosamidin. Bacterial chitinases, as well as

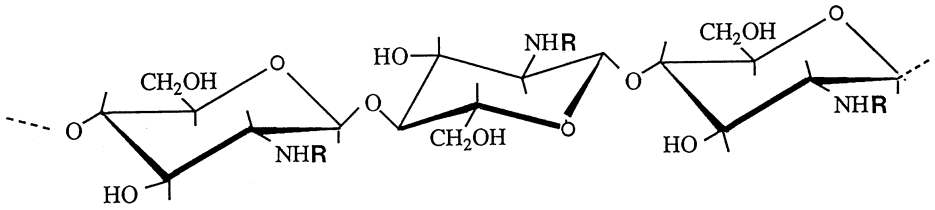

Figure 1. The repeating unit in chitosan is glucosamine (R = H). Some repeating units are however acetylated randomly (R = $COCH_3$) and each chitosan has a degree of acetylation value. The degree of acetylation is the molar fraction of *N*-acetylglucosamine.

hevamine, retain the anomeric configuration of the substrate [14–17]. Their fold is $(\alpha\beta)_8$-barrel with a catalytic groove. **Family 19** includes plant chitinases only, one subgroup of which has a N-terminal cysteine-rich domain. They are inverting enzymes and are not inhibited by allosamidin. Their fold resembles that of lysozyme [18]; lysozyme is a retaining enzyme, however. **Family 20** includes bacterial chitobiases active on chitobiose, and eukaryotic hexosaminidases. Their fold is $(\alpha\beta)_8$-barrel, with a catalytic pocket instead of a groove [19].

Lysozyme

Lysozymes fall into sequence families 22–24, nevertheless lysozymes and endochitinases of family 19 are parts of a superfamily. Lysozymes in most cases are both chitinases and muramidases [15], i.e. hydrolyse chitins and the bacterial cell wall $\beta(1-4)$-linked alternate copolymer of *N*-acetylglucosamine and *N*-acetylmuramic acid. Lysozymes accommodate up to 6 adjacent pyranose rings that interact with sites A to F. The overall structure and the active site geometry of human and avian lysozymes are similar. The hydrolysis reaction proceeds via proton transfer from the carboxyl group of Glu-35 to the substrate and then a hydroxyl group is added to the anomeric carbon. The structure of the active site cleft of human lysozyme has been elucidated [20]. Present understanding explains the immediate splitting of the hexamer into tetramer and dimer in terms of the role of bound water molecules in the binding site D.

Chicken and hen egg white lysozymes in vitro exert maximum activity on chitosan with degrees of acetylation close to 0.20 [21, 22]. Lysozyme is ineffective on fully deacetylated chitosan and very poorly effective on α-chitins [23]. One would therefore speculate that chitosans with degree of acetylation 0.20 would be the most suited to the manufacture of wound dressings, but the non-woven dressings manufactured from chitin after spinning chitin solutions in dimethylformamide are widely used and provide good results; the same can be said for a variety of modified chitosans [24]. More than one hydrolase, therefore, should be active on chitins and chitosans.

Chitinase

An endoglucosaminidase (4-methylumbelliferyl-tetra-*N*-acetyl chototetraoside hydrolase) is known to hydrolyse tritiated colloidal chitin [25] as well as the artificial substrate 4-methylumbelliferyl-$[GlcNAc]_4$, but not 4-methylumbelliferyl-β-*N*-acetylglucosamine, the substrate for exo β-*N*-acetylhexosaminidase [26, 27]. This enzyme, present in human serum and leukocytes, is in fact a chitinase [28]. Its differences from lysozyme have been studied [29]. It has been purified 56,000-fold and eluted at a different position than lysozyme.

The endoglucosaminidase behaves as a chitinase because 1) it liberates dimer and trimer from methylumbelliferyl-$(GlcNAc)_3$; 2) it hydrolyses colloidal chitin, chitin azure and glycolchitin; 3) it is inhibited by allosamidin, an inhibitor of family 18 chitinases; 4) it exerts no hydrolytic action on *Micrococcus lysodeikticus* cell walls, a substrate for lysozyme; 5) chitin serves as affinity sorbent for separation from accompanying proteins [26, 27].

Macrophages are capable of producing very large amounts of the enzyme under specific circumstances. About 1% of the secretory proteins of these cells is chitinase [30]. The stress exerted by the glucosylceramide accumulation in macrophages of Gaucher patients favours expression of chitotriosidase.

This inherited deficiency in glucocerebrosidase activity causes lysosomal storage of glucosylceramide in macrophages. The endoglucosaminidase was purified 3600-fold from spleen extracts and sequenced. The first 22 amino acids of the N-terminus as well as 21 of an intramolecular segment, correlated with members of family 18 of glycosyl hydrolases, reported in table 1 [31, 32]. Escott and Adams showed experimentally that on differentiation of the monocytes into macrophages in vitro, chitinase is released into the growth medium [25]. Indeed, it seems likely that chitotriosidase activities [31] and the chitinase activities [32] are in fact the same. It is at present unclear whether the activites are due to cell differentiation into macrophage-like cells, or whether the production of the enzymes is a direct response by the monocytes to the presence of chitin in the medium [25]. Two isoforms were isolated with isoelectric points 7.2 and 8.0 and molecular masses 50 and 39 kDa, respectively. The cDNA encoding chitotriosidase was cloned [30]: the nucleotide sequence of the cloned cDNA predicted a protein with amino acid sequences

Table 1. Alignment of putative active site regions in members of family 18 of glysosyl hydrolases.

Endowed with enzymatic activity	Active site region
Human chitinase, from macrophages, chitotriosidase	FDGLDLDWEYP
Chitinase from virus *Autographa californica*	FDGVDIDWEFP
Chitinase from nematode *Brugia malayi*	FDGFDLDWEYP
Chitinase from insect *Manduca sexta*	FDGLDLDWEYP
Chitinase from fungus *Aphanocladium album*	FDGIDIDWEYP
Chitinase from fungus *Trichoderma harzianum*	FDGIDVDWEYP
Chitinase A1 from procaryote *Bacillus circulans*	FDGVDLDWEYP
Chitinase from plant *Nicotiana tabacum* class v	FHGLDLDWEYP
Devoid of enzymatic activity	
Human cartilage glycoprotein, *tabacum* class v	FDGLDLAWLYP
Human glycoprotein, from oviduct	FDGLDLFFLYP
Murine YM-1, from murine macrophages	FDGLNLDWQYP

Adapted from Boot et al., cf. ref. 30.

identical to those established by peptide sequencing [33]. Overdijk et al. have also shown that chitinase activity levels in guinea pigs increase upon infection with *Aspergillus fumigatus*, thus substantiating the hypothesis of the role of chitinase in a defence system [34]. Chitinase activity was found to be associated with THP-1 (a monocyte cell line) following culture with colloidal chitin [35].

Chitinase-related proteins

A number of mammalian proteins exist which are structurally similar to family 18 chitinases (table 1), but which are devoid of glycosidase activity against chitinase substrates, having E in the active site WEYP sequence replaced by other aminoacids. They may however utilize structural elements typical for chitinases to mediate binding to specific carbohydrates. This is the case for estrogen-dependent secretory proteins from sheep, human, baboon, cow, pig and hamster, expressed in a temporally and regionally specific manner in the oviducts at the time of fertilization and embryo development. Alignments of their aminoacid sequences showed various degrees of identity (from 26 to 36%) to chitinase [36].

mRNA for human cartilage gp-39 is present in human articular chondrocytes as well as in liver, but not in muscle, lung, pancreas, mononuclear cells or fibroblasts. It was detected in synovial specimens and in cartilage obtained from patients with rheumatoid arthritis.

The mammalian proteins are found in situations where tissue remodelling occurs. The bovine oviduct glycoprotein and the whey protein, with sequences similar to those specified in table 1, are secreted in increased amounts in parallel with involution of the mammary gland following cessation of lactation. The synthesis and secretion of the estrogen-induced oviduct-specific glycoprotein appears to correlate with morphological and functional changes occurring prior and during ovulation. Turnover and degradation of extracellular matrix components is also elevated in the rheumatoid joint [37]. Therefore, human chitinase should be seen in the context of an array of chitinase-like proteins present in the human body.

Hexosaminidases

Hexosaminidases (3.2.1.52, family 20) cleave β-glycosides of both *N*-acetylglucosamine and *N*-acetyl-D-galactosamine, and hydrolyse gangliosides in higher organisms. Deficiencies in these enzymes result in gangliosidoses such as Tay-Sachs and Sandhoff diseases [19]. Sequence identities between *Serratia* chitobiase and other family 20 eukaryotic hexosaminidases are 16–54% [8]. *N*-Acetyl-β-D-glucosaminidases (EC 3.2.1.30) hydrolyse terminal nonreducing 2-acetamido-2-deoxy-β-glucose units in chitobiose and higher analogues, and in glycoproteins; they are part of the defence mechanism of vertebrates and elevation of their levels is obtained by administration of chitosan in a number of ways [7].

Nonspecific activities

Chitosans with varying degrees of acetylation and substitution have been found to be susceptible to a variety of hydrolases. For example, human, plant and fungal α-amylases, and animal, plant and recombinant lipases have been found to hydrolyse chitosans [38]. At the present time we do not know whether human lipase shares this property with the corresponding enzymes from other sources, but we know for sure that chitosans are more vulnerable than expected by the nonspecific actions of a number of enzymes. Chitosan-based wound dressings are depolymerized by lyzozyme and *N*-acetyl-β-D-glucosaminidase. The action of these enzymes permits resorption of the dressing and liberation of GlcNAc, glucosamine and their oligomers which exert beneficial actions such as the activation of macrophages and in part are incorporated into dermatan sulfate, chondroitin sulfate and hyaluronic acid. Vascularization of the newly formed tissues, presence of normal cellular elements, and ordered deposition of collagen fibres are prominent aspects of the resulting scars.

The hydrolases described above presumably contribute to the depolymerization of chitin-based dressings in vivo and their presence should also be considered when chitosan items are involved. Lysozyme, a defensive enzyme against pathogens endowed with chitosanase-like activity, lipases, presumed to be nonspecifically active on chitosans, and *N*-acetyl-β-D-glucosaminidase, a lysosomal glycosidase involved in degradation of asparagine-linked glycoproteins [39] also capable of hydrolysing chitooligomers, and the human chitinase, a secretory protein of macrophages, are the enzymes which, according to our present knowledge, control the biological effects and fate of chitin-based dressings and drug carriers by partial depolymerization, release of oligomers and monomers and, eventually, total resorption.

Insight into the catalytic mechanism of plant chitinases of both families 18 (classes III and V) and 19 (classes I, II and IV) has been promoted by structural analysis of the class II barley endochitinase and the class III hevamine, which are family representatives as far as the known three-dimensional structures of chitinases are concerned [40]. The structural similarity between the barley endochitinase and hen egg white lysozyme was used to pinpoint the positional correspondence between conserved Glu-67 of barley chitinase and the essential catalytic Glu-35 of hen egg white lysozyme [18]. The catalytic mechanism offers two possibilities: retention or

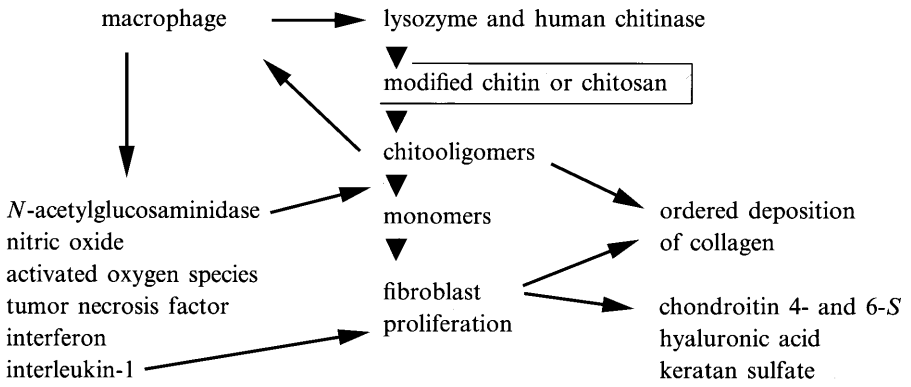

Figure 2. Scheme of the interactions between exogenous chitins/chitosans and human cells in a wound. Lysozyme, normally produced by macrophages, hydrolyses susceptible modified chitins/chitosans to oligomers, which activate macrophages to produce nitric oxide, activated oxygen species, tumor necrosis factor-α, interferon and interleukin-1. Activated macrophages increase their production of lysozyme, chitinase and *N*-acetyl-β-D-glucosaminidase, which catalyse the total depolymerization to monomers. The monomeric aminosugars become available to fibroblasts which proliferate under the action of interleukin-1, for incorporation into chondroitin 4- and 6-sulfate, hyaluronan and keratan sulfate, thus guiding the ordered deposition of collagen, also influenced by chitooligomers (ref. 6).

inversion of the anomeric configuration. When the carboxylate base is too far from the catalytic protonated acidic function, inversion takes place. The β-configuration is retained when the catalytic Glu is assisted by an anionic carboxylate or by the substrate itself (anchimeric assistance of the *N*-acetyl group). These situations show that only one enzyme function may be sufficient to perform the catalytic step and that, in other words, such a simple mechanism could be activated by hydrolases other than chitinases and lysozymes that might come into contact with chitosan.

Effects of chitooligomers on wound healing processes

The hydrolytic actions of lysozyme, chitinase and *N*-acetyl-β-D-glucosaminidases, which produce chitooligomers capable not only of macrophages activating and promoting collagen deposition, but also of being incorporated into extracellular matrix components, appear to be the key to the activities exerted by chitosans in the rebuilding of physiologically normal tissues (fig. 2) [41].

The secretion of enzymes, interleukins, tumor necrosis factor, nitric oxide, peroxide and other compounds by activated macrophages provides macroscopic evidence of the biochemical significance of chitosans (fig. 2). These are peculiar features of modified chitins and chitosans: in fact, acetamidodeoxycellulose and aminodeoxycellulose, semisynthetic analogues of chitin and chitosan, respectively, are not hydrolysed by lysozyme and chitinases and do not induce these responses.

Various chitosans were found to induce production of tumour necrosis factor-α (TNF-α) in human monocytes [42]. Their ability to induce TNF-α was dependent on their molecular weight. Lipopolysaccharides and water-soluble chitosans recognize a binding site on monocytes which involves CD14, a receptor of lipopolysaccharides also present in macrophages. These data were obtained by using biotinylated chitosan on cells pre-treated with lipopolysaccharides, polyuronates, hyaluronic acid and antibodies. They suggest that the CD14 receptor, involved in cytokine production, recognizes chitosan, presumably due to its similarity with the saccharide portion of lipid A despite the 1–6 anhydroglucosidic link (instead of 1–4 for chitosan). The presence of receptors for GlcNAc-glycoproteins on macrophages was reported [43]. This not only provides an explanation of the immunoadjuvant activity of chitosan, but also of the wound healing mechanism based on macrophage activation. Chitooligosaccharide derivatives containing N-linked side chains which mimic the lipopolysaccharides were found to possess immunostimulating and antitumour activities [44].

The combination of basic fibroblast growth factor (bFGF) and methylpyrrolidinone chitosan has a beneficial influence on impaired wound healing because methylpyrrolidinone chitosan prevents excessive scar formation (fig. 3) and bFGF induces faster wound closure [45]. The incorporation of bFGF into a chitosan spongy fleece allows the homogeneous distribution of the factor over the entire wound area with a minimum of irritation. The methylpyrrolidinone chitosan gels immediately upon contact with physiological fluids and is well tolerated in full thickness wounds. This is an important advantage over the application of ointments, gels or solutions containing bFGF. Lysozyme in the wound gradually depolymerizes methylpyrrolidinone chitosan and provides the sustained delivery of bFGF and *N*-acetylglucosamine [46]. It might even be unnecessary to remove the wound dressing during or after treatment. Slow healing wounds could therefore be treated with bFGF-loaded methylpyrrolidinone chitosan over a period of 48–72 h for maximum release of the bFGF.

Lysozyme, human chitinase and *N*-acetylglucosaminidase promote metabolic degradation of exogenous modified chitins: the oligomers stimulate various cells, while the monomers are phosphorylated and incorporated into hyaluronate, keratan sulfate and chondroitin sulfate. The GlcNAc present in chitosan is responsible for nitric oxide production by rat peritoneal exudate macrophages [47] at levels tolerable in terms of inflammatory response but effective in terms of repair of wound tissue or tumoricidal action.

Applications of chitosan to wound tissues are therefore beneficial due to the action of these hydrolases, which are essentially expressed as a defence against pathogens and dysfunction. In the case of bone regeneration, however, we still know little about the mechanism of action of chitosan and the possible effect on enzymatic activities.

Nitric oxide production after macrophage activation is receiving increasing attention. Nitric oxide is produced enzymatically via nitroso-L-arginine, a process requiring superoxide ions. The nitric oxide synthase carries both a heme and a cytochrome Fe^{2+} group, and is NO-inhibited. Nitric oxide can also be produced chemically from primary amines in the ferrous/ferric peroxidative mechanism. Nitric oxide enhances phagocytic activity against bacteria and neoplastic cells, and is believed to act as a synchronizing messenger because it simultaneously affects several unrelated biochemical processes, including the cytochrome chain [48]. In fact, the activities of most iron-carrying enzymes including the cytochrome metabolic chain, peroxidase, catalase, xanthine oxidase, aldehyde oxidase and succinate dehydrogenase, are affected as a consequence of the formation of nitrosyl complexes. Nitric oxide diffuses easily through both hydrophilic and hydrophobic media and penetrates into the active site inside a folded metallo-protein. It is also a neurotransmitter and a major vasodilator. Its chemical reactivity is shown by the instant formation of nitrosyl complexes, nitrite and nitrosonium salts; its reactions with amines are well known.

An example of synchronization of interest in this context is the NO-induced vasodilation which increases the blood supply through arterioles to the affected area, and with it the influx of monocytes and macrophages plus oxygen and nutrients. The vasodilation of veins minimizes acidosis and buildup of wastes; at the same time nitric oxide enhances the mobility of the macrophages and therefore the phagocytic process.

The consequences of chitosan application to living tissues could possibly go far beyond the enhanced production of NO by macrophages; additional NO could be produced chemically, but some could be consumed as nitrite by the chitosan amino groups. Thus, extra amounts of NO could be beneficial and help resorb chitosan. The presence of chitosan could presumably have some as yet undocumented consequence on the iron-enzymes.

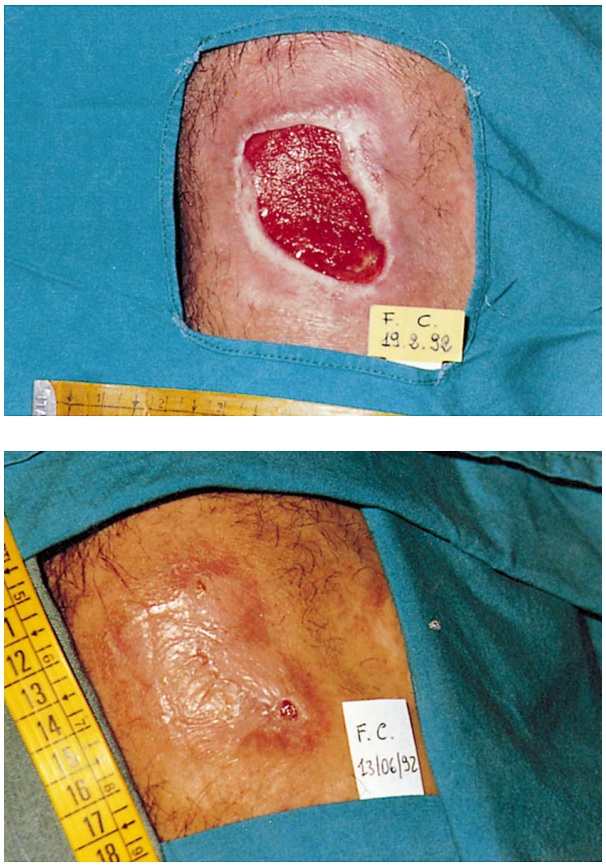

Figure 3. Healing of a congenital ulcer in a 20-year old patient. The ulcer was dressed with methylpyrrolidinone chitosan, with frequent changes (8 February 1992), and a few days later (19 February 1992) (upper photograph) early signs of healing were observed in the peripheral area; (lower photograph) complete healing was observed on 13 June 1992. Dimensions in centimeters. (From Muzzarelli R. A. A. and Biagini G., in ref. 7.)

Ordered reconstruction of soft tissues

Chitin-based products for wound dressing today include: regenerated chitin powders, chitin non-woven fabrics, porous beads, lyophilized soft fleeces, gel-forming lyophilized soft fleeces and gauzes, laminated sheets, double-face laminated sheets, transparent films, microspheres, and combinations with other polymers such as cellulose, collagen, keratin, chondroitin sulfate, polyester, poly(tetrafluoroethylene), polyurethane and polyethylene terephthalate.

In general, these items provide improved healing of surgical wounds by first intention in all cases. Activated macrophages and fresh neutrophils are more abundant than in controls; fibroblast activation is also intense, and in no case was suppuration or microbial proliferation reported. Vascularization and the presence of regular cellular elements provide smooth scars.

In a skin substitute made of collagen I and III and chondroitins 4- and 6-sulfate (72% collagen, 20% chitosan and 8% chondroitins 4- and 6-sulfate), the cross-links between the primary amine and the sulfate groups

were essential in imparting insolubility and mechanical resistance in the course of cell culture. The skin substitute included the human fibroblasts on the 'dermal' side, and the keratinocytes on the 'epidermal' side. The latter, once exposed to air during the last stage of the culture, generated a skin similar to the natural one in which the epidermal layers, including the stratum corneum, were present. The differentiation processes included the expression of filaggrin and keratin and the formation of a structure similar to the basal lamina. Chitosan was found to be indispensable not only for the insolubility, but also for increasing the production of collagen and factors by fibroblasts. The addition of chitosan also increased the cytotoxicity levels, and provided good adhesion (better than on collagen alone) without proliferation problems. The dermal substrate did not present any antigenic incompatibility; it could be easily stored for emergency and was free of viral contamination. The desired porosity (0.05–0.10 mm) was obtained by selecting the lyophilization conditions. This dermal substitute allowed controlled vascularization and fibroblastic colonization. Fibroblasts synthesized the different macromolecules of the dermal extracellular matrix in an organized manner and limited formation of granulation tissues and hypertrophic scars [49–51].

Chitosan is suitable as a matrix for anchorage-dependent mammalian cell encapsulation [52]. Macrocapsules made of polyacrylonitrile-polyvinyl chloride (PAN-PVC) and hollow fibres (of interest in neurosurgery) were filled with a PC12 cell suspension in chitosan solution. After sealing the hollow fibre, chitosan was precipitated by dialysis in physiological saline. The release of nerve growth factor (NGF) from R208N.8 fibroblasts was assessed according to the induction of neuritic outgrowth from PC12 cells. The chitosan prevented extensive cell clumping and necrosis (which is known to take place in alginate gels and other encapsulation devices). When macroencapsulated with chitosan, the PC12 cells appeared to attach successfully to the precipitated chitosan and responded to exposure to NGF by extending neurites. Differentiation of neuronal cells was also supported by the chitosan matrix. These encapsulated PC12 cells were implanted into the basal ganglia of monkeys: 4 weeks following implantation in capsules containing the chitosan matrix, cells were viable and small clusters were distributed along the hollow fibre length, while in the absence of chitosan they were aggregated in large clusters with necrotic cores.

The in vitro biocompatibility of wound dressing in terms of toxicity for fibroblast was assessed for chitosan lactate, glutamate and chloride salts, for methylpyrrolidinone chitosan and for three commercial wound dressings made of collagen, alginate and gelatin. Methylpyrrolidinone chitosan and collagen were the most compatible materials [53].

Chitosan was associated not only with other biopolymers but also with synthetic polymers, for instance polyvinyl alcohol and polyurethane aqueous dispersions, to produce wound dressings [54]. Biosynthetic wound dressings with a drug delivery capability composed of a spongy sheet of chitosan and collagen, laminated with a gentamycin sulfate-impregnated polyurethane membrane, were produced. From in vitro evaluation, it was shown that this wound dressing is capable of suppressing bacterial growth and minimizing cellular damage. This wound dressing was clinically evaluated on superficial second-degree burns, deep second-degree burns, donor sites and pressure sores: it achieved good or excellent results in 97% of the patients.

Regeneration of bone tissues

Bone defects, surgically produced in sheep and rabbit models, were treated with freeze-dried imidazolyl chitosan and methylpyrrolidinone chitosan [55–58] (fig. 4). Histological observations 60 days after surgery showed a considerable presence of neoformed bone tissue, as opposed to controls, originating from the pre-existing bone as well as from the periosteum. The cationic nature and the chelating ability of the methylpyrrolidinone chitosan apparently favoured mineralization. Endosteal-periosteal and bone marrow osteoblast-like precursors, stimulated by growth factors entrapped in the coagulum-polysaccharide mixture, gave rise to intramembranous bone formation. Ultrastructural examination showed that bone osteoid formation was followed by mineralization of the tissue. Osteoinduction was also observed in rabbit endochondral bones [59].

Various studies dealing with the reconstruction of the parodontal tissue with chitosan [60, 61] were a prelude to the discovery of the osteoinductive activity of chitosan. Surgical wounds from wisdom tooth avulsions were treated with freeze-dried methylpyrrolidinone chitosan, which promoted osteoconduction. The space left after avulsion was filled with newly formed bone tissue, which conferred desirable mechanical and physiological characteristics to the healed wound site. Morphological evidence obtained from biopsies confirmed the radiographic data. Methylpyrrolidinone chitosan was progressively depolymerized by lysozyme and was no longer detected 6 months after surgery. Methylpyrrolidinone chitosan was found useful in apicectomies as well. None of the patients reported adverse effects over 3 years of observation [56].

Anti-inflammatory drug delivery

Indomethacin has been administered per os to male humans and dogs in the form of rapid-release granules and slow-release beads manufactured with chitosans of

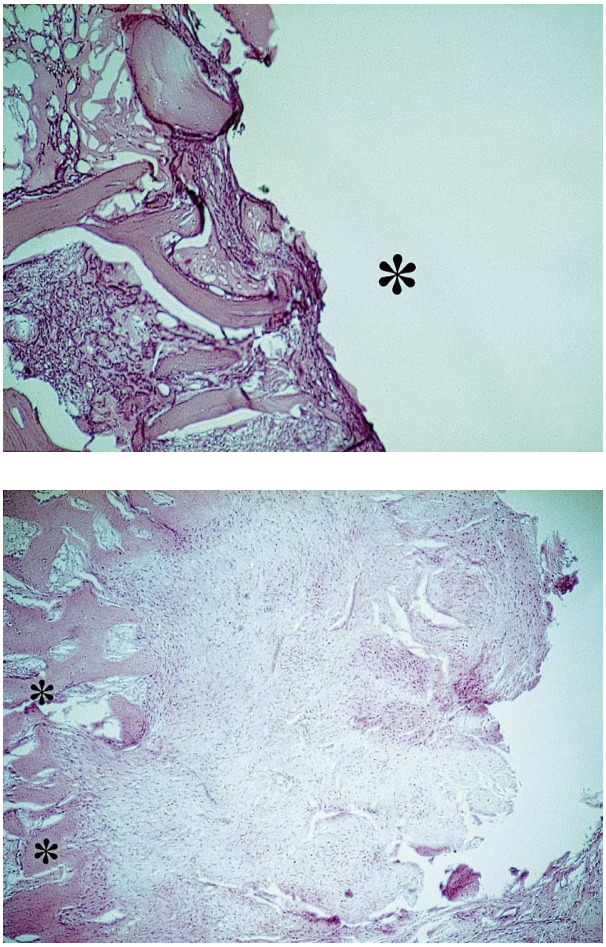

Figure 4. Healing of a surgical lesion in sheep, with the aid of imidazolyl chitosan. Upper photograph: Control, 40 days after surgery. No neoformed osseous tissue nor reactive fibrotic tissue filling the 7–mm hole (*) is present in the experimental lesion. Lower photograph: Imidazolyl chitosan-treated wound, 40 days after surgery. Advanced healing of the experimental lesion is taking place via structurally organized osseous trabeculae (**), adjacent to an ample nodular formation undergoing histo-organization. (R. A. A. Muzzarelli and G. Biagini, original results; see also ref. 57.)

defined molecular weight [62]. By mixing the two preparations in suitable proportions, optimization in vivo and in vitro was achieved [63, 64]. Indomethacin, an acidic compound, is an anti-inflammatory drug which inhibits the enzymes involved in the epoxidation of arachidonic acid for prostaglandin synthesis. Preliminary to the preparation of the drug-excipient association, the adsorptive capacity was found to rank in the order chitosan > chitin > microcrystalline cellulose [65]. The solubility of indomethacin was enhanced with increasing concentrations of 3800 Da chitosan, and the oral absorption rate from this kneaded mixture was improved compared to indomethacin alone [66]. The kneaded product was soluble in water at 37 °C and exhibited a typical X-ray diffraction spectrum totally different from the drug or drug + chitosan mixture spectra. It was concluded that kneading promotes formation of a soluble complex mainly based on ionic and hydrophobic interactions. While this point was not explicitly examined in detail, it is believed that the delayed release of drugs is controlled by the susceptibility of the chitosan-drug complex to lysozyme.

Glucosamine itself is an anti-inflammatory drug: it is synthesized in the human body from glucose and incorporated into glycosaminoglycans and glycoproteins. It has been administered to human volunteers by intravenous, intramuscular and oral routes for pharmacokinetic studies [67]. The amount administered was 250–400 mg glucosamine sulfate. Exogenous glucosamine sulfate salt is currently used for the therapy of osteoarthritic disorders [67] (Dona (R), manufactured by Rotta, Monza, Italy).

Glucosamine diffuses very rapidly in most tissues and organs, even after oral administration, and it accumulates in the articular tissue and in the bone. The rapid diffusion is due to its low molecular weight, its solubility in water and its pK_a of 6.8, which means that at the pH of the blood (7.4) 25% of glucosamine is ionized and 75% is non-ionized [67, 68].

Anticholesterolemic dietary foods

Chitosan binds fatty acids to form the corresponding complex salts that bind triglicerides, fatty and bile acids, cholesterol and other sterols, and a great portion of these bound lipids are excreted [69, 70]. Bound triglycerides escape hydrolysis by lipase. Hydrochloric acid in the stomach appears not to hydrolyse chitosan fatty acid salts because this material hardly gets wet. Rather, this chitinous material grows in size as it travels through the gastrointestinal tract. Studies on rats indicated that the hypocholesterolemic action of chitosans is independent of their molecular weight within the tested viscosity range. Judging from the ineffectiveness of the glucosamine, a certain degree of polymerization is required to provoke a cholesterol-lowering activity [71]. The hydrolysates with average molecular weights above 10 kDa were more effective in enhancing fecal excretion of neutral steroids [72]. The effects on the composition of neutral steroids were diverse, depending on the preparations [42, 73–75].

The consequences of chitosan administration on 3-hydroxy-3-methylglutaryl CoA reductase in rats fed a sterol diet were studied [76]. This enzyme is the key regulatory enzyme of cholesterogenesis and produces mevalonic acid, a precursor of cholesterol. The enzyme levels remained close to the normal value with a 7.5% chitosan formula, and plasma high density lipoprotein cholesterol did not decrease. On the other hand, the enzyme activity was overstimulated in the sterol + cholestyramine group, where livers were smaller. The advantages of chitosan over cholestyramine, a commer-

cial anticholesterolemic resin, have been clearly described, but the nexus between chitosan and the enzyme has not been elucidated. For example, one might hypothesize that enhanced availability of glucosamine would have consequences on the oxysterols which regulate 3-hydroxy-3-methylglutaryl CoA reductase in human fibroblasts and leukocytes [77].

Data on the dietary effects of chitosan in adult men were published [37]. The chitosan in this study was administered in the form of biscuits over a period of 4 weeks. When chitosan was given in the diet (3–6 g/day), the serum total cholesterol level significantly decreased, while the serum high density lipoprotein-cholesterol level significantly increased. Similar results have been obtained with rats raised for 20 days after dietary administration of chitosan [78]. The results on humans show a favourable effect with a very low dose within a short period, and are thus worthy of special attention [79].

The chitosan intake (biscuits) by healthy volunteers was found to produce a significant decrease of the faecal phenols, p-cresol and indole, in analogy with other polysaccharides [80]. Chitosan inhibited the putrefactive activity of the intestinal microbiota, thus reducing the risk of disease states.

Tablets containing chitosan (400 mg), guar gum, ascorbic acid and micronutrients are available (Nofat (R), manufactured by SIRC, Caleppio, Italy). Eighty obese adult subjects with hyperlipidemia were treated with a hypocaloric diet plus 4 tablets/day of chitosan dietary fibre or with a hypocaloric diet plus 4 tablets/day of placebo for 4 weeks. At the end of the study period a statistically significant reduction in the body weight and overweight, triglycerides and total and LDL cholesterol, and an augmentation of HDL cholesterol were observed in both groups but in the chitosan treated group the differences were statistically greater than in the placebo group [81]. Similar results were obtained by other authors [43]. Similar tablets containing also *Garcinia cambogia* hydroxycitrate are available (Colenon®, manufactured by SIRC, Caleppio, Italy).

The basicity of the amino group, on which the antiulcer activity of chitosan is based [82–84], is the key to the biological activity of this application as well.

Discussion and conclusions

As the present data show, the applications of exogenous chitosan items for therapeutic purposes in general lead to the liberation of monomers and oligomers that exert beneficial effects. While chitosan has been found to be a substrate for many enzymes of industrial and general interest, little is known about enzymes of human origin, such as collagenases and heparinase. The healing of cartilagenous and bone tissues with chitosans should involve a range of enzymes.

The oral administration of chitosan seems to lead to partial hydrolysis and generation of monomers. As seen in the above description of current studies on the anticholesterolemic action of chitosan, there is reasonable though incomplete evidence that digestive enzymes, mainly pancreatic lipase, would be able to hydrolyse chitosan. Human lipases should be used to confirm the data obtained with recombinant, plant and porcine lipases [85, 86].

An example underlining the importance of enhanced levels of glucosamine as a possible consequence of chitosan hydrolysis by human digestive enzymes, is the inhibition of glucokinase and impairment of insulin secretion. Glucosamine (600 mg per kg body weight injected over 30 s) impaired insulin secretion in response to glucose in rats, probably because of inhibition of glucokinase [87]. Glucosamine has high affinity for hepatic glucokinase and slow rate of phosphorylation. Glucokinase is important for glucose homeostasis during hyperglycemia when hexokinase is inhibited by high intracellular glucose 6-phosphate concentration. Nevertheless, the amounts of glucosamine used for these experiments were fairly high (only 4.5 mg/kg per day were used intravenously in rabbits to demonstrate enhancement of serum lysozyme) and would largely exceed those (3–6 g chitosan/day) used on humans [79].

The generation of glucosamine from chitosan by human enzymes, and the role of the exogenous glucosamine on various enzymatic activities are important subjects which demand further research.

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