Peritoneal application of chitosan and UV-cross-linkable chitosan

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Received 31 August 2005; revised 2 December 2005; accepted 16 December 2005
Published online 31 May 2006 in Wiley InterScience (www.interscience.wiley.com). DOI: 10.1002/jbm.a.30740

Abstract: The suitability of chitosan and UV-cross-linkable chitosan for intraperitoneal use, for example as a barrier device for preventing peritoneal adhesions or for drug delivery, was examined. *In vitro* experiments using two major cell types present in the peritoneal cavity (mesothelial cells and peritoneal macrophages) revealed neither attractive interactions between cross-linked chitosan gels and the cells nor a proliferative effect. However, the same UV-cross-linked chitosan applied in the peritoneal cavity of rabbits caused a granulomatous reaction with adhesion formation within two weeks in all animals, which persisted up to 4 weeks after exposure. Unmodified chitosan also caused adhesions, while UV irradiation did not. UV-cross-linkable chitosan induced significant elevations in MIP-2 and TNF-α from peritoneal macrophages, suggesting that soluble mediators could play a role in inducing adhesion formation. These results reinforce the view that the predictive value of *in vitro* cytotoxicity assays in matters of biocompatibility may not be sufficient, and suggest that other assays such as cytokine levels may be of value in predicting outcomes in situations involving multiple cell types (i.e. *in vivo*). © 2006 Wiley Periodicals, Inc. J Biomed Mater Res 78A: 668–675, 2006

Key words: chitosan; UV-cross-linkable; peritoneum; adhesion; biocompatibility

INTRODUCTION

Postsurgical adhesions are common complications of abdominal surgery, which can cause significant postoperative morbidity such as severe abdominal and pelvic pain, infertility, and bowel obstruction.[1] Numerous physical barrier devices have been developed to prevent peritoneal adhesions, using complex polysaccharides and other biomaterials.[2–7]

One such polysaccharide is chitosan, a linear copolymer of glucosamine and *N*-acetylglucosamine, obtained by partial N-deacetylation (>50%) of the natural polymer chitin. Chitosan has been reported to be biocompatible,[8,9] and its degradation products are nontoxic and nonimmunogenic.[10] In addition, chitosan is a cationic polymer with bioadhesiveness mediated by ionic interactions between positively charged amino groups of the polymer and negatively charged mucosal surfaces.[11] For these reasons, chitosan has attracted interest in a broad range of biomedical areas such as wound healing,[12,13] surgical adhesives,[14] muco-adhesive oral drug delivery,[15] gene delivery,[16] and tissue engineering.[17,18]

Chitosan's biocompatibility and bioadhesiveness also make it attractive for intraperitoneal drug delivery and in preventing peritoneal adhesions, where it is helpful for a material to remain adherent at the applied location during an extended healing process. Chitosan-based materials such as carboxymethyl chitosans[19,20] or chitosan/alginate mixtures[21] have been considered in patents as potential postsurgical adhesion barrier materials. However, there is little published on this application of chitosan outside of the patent literature.[19–22] One patent claimed that postsurgical lavage with *N*,*O*-carboxymethyl chitosan significantly reduced adhesions in a rat cecal abrasion mod-

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Contract grant sponsor: DuPont/MIT Alliance
Contract grant sponsor: NIGMS; contract grant number: GM073626

el.[20] This is in contrast to a report in which repeated injections of chitin or chitosan resulted in intraperitoneal inflammation (but no adhesions).[23] Here we attempt to clarify the usefulness of chitosan and a UV-cross-linkable chitosan for intraperitoneal applications such as drug delivery and as a barrier device for preventing peritoneal adhesions.

MATERIALS AND METHODS

Materials

Chitosan glutamate (Protasan UP G113; Mw, <200 kDa; degree of deacetylation, 75–90%) was purchased from Novamatrix (Norway). 4-Azidobenzoic acid was purchased from TCI America (Portland, OR). All other reagents were purchased from Sigma-Aldrich (St. Louis, MO) unless specified otherwise.

Synthesis and characterization of the UV-cross-linkable chitosan

UV-cross-linkable chitosan (Az-chitosan) was synthesized based on modification of a previously reported method.[24] Briefly, 200 mg (1.24 mmol) of chitosan glutamate (chitosan) was dissolved in 15 mL distilled water in a round bottom flask. *N*,*N*,*N*′,*N*′-Tetramethylethylenediamine (116.2 mg) was dissolved in 1 mL distilled water and added to the chitosan solution. 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide 70 mg (0.451 mmol) and 4-azidobenzoic acid 40 mg (0.245 mmol) were dissolved in 1 mL of distilled water and dimethyl sulfoxide, respectively, and briefly mixed prior to addition to the chitosan solution. The reaction was conducted at pH 5 and room temperature overnight. The modified chitosan was purified by ultrafiltration using a YM10k membrane (MWCO: 10,000). For sterilization, the solution was filtered using 0.22 μm aseptic filter and lyophilized while maintaining sterility. The macromonomer solution (20 mg/mL in saline) was polymerized into a gel with exposure to longwave UV irradiation (Black-Ray, UVP, radiation range 315–400 nm, peak at 365 nm) for 1 min. The chemical structure of Az-chitosan was determined using NMR and a UV absorbance profile. The morphology of Az-chitosan gel (cross-linked) was examined by scanning electron microscope (JEOL JSM 6060, JEOL USA, Peabody, MA) and compared with lyophilized Az-chitosan (not cross-linked) and unmodified chitosan. The samples were sputter-coated with palladium and gold (100 Å thick) prior to observation.

Cell culture

Mesothelial cells and peritoneal macrophages, the two major cells present in the peritoneal cavity,[25] were used for *in vitro* experiments. Human mesothelial cells (ATCC, CRL-9444) were cultured in Medium 199, containing Earle's salts, L-glutamine, and 2.2 g/L sodium bicarbonate and supplemented with 3.3 n*M* epidermal growth factor, 400 n*M* hydrocortisone, 870 n*M* insulin, 20 m*M* HEPES, and 10% fetal bovine serum (Invitrogen). Mouse peritoneal macrophages (ATCC, CRL-2457) were cultured as a suspension in Dulbecco's modified Eagle's medium (DMEM) containing 4 m*M* L-glutamine, 1.5 g/L sodium bicarbonate, 4.5 g/L glucose and supplemented with 5 m*M* HEPES and 5% fetal bovine serum (ATCC). Cells from passage 5 through 25 were used for the following studies.

Cell–gel interaction

A thin film of chitosan gel was prepared on tissue-culture treated petri dishes (100 × 20 mm^2): 200 μL of Az-chitosan saline solution (20 mg/mL) was placed on a petri dish rotating at 3000 rpm on a spincoater (Spincoater P6700, Specialty Coating Systems, Indianapolis, IN) and polymerized under UV irradiation. Stripes of gel were formed toward the periphery of the petri dish. The gel was washed with sterile phosphate-buffered saline three times. Cell suspension (10 mL; 50,000 cells per 10 mL) was then added to the gel-coated petri dish, and cell growth was observed daily using an optical microscope (Axiovert 200, Zeiss, Thornwood, NY). Alternatively, cells were grown in a well of 24-well plate, which was completely coated with Az-chitosan gel, and compared with those grown in an uncoated well.

In vitro cytotoxicity test

Cells were seeded into 24-well plates at a density of 50,000 cells per well in 1 mL of culture media and left overnight for attachment. Solution of chitosan or Az-chitosan (100 μL) in saline was added to each well to bring the final chitosan concentration in culture media to 0.2 or 2 mg/mL. To a control group, 100 μL of saline was added in lieu of chitosan solutions. Cells were also cultured on a 100 μL Az-chitosan gel (the same amount of chitosan per well as added with the 2 mg/mL solution) UV-cross-linked prior to addition of cells. Cytotoxicity of the materials was assessed with a commercially available MTT viability assay kit (Promega CellTiter 96 Nonradioactive Cell Proliferation Assay). After 1, 3, and 7 days (or 5 days for macrophages because of excessive cell growth at later time points), 150 μL of the MTT reagent (tetrazolium salt solution) was added to the wells, and the plate was placed in an incubator at 37°C for 4 h. The purple formazan produced by active mitochondria was solubilized using 1 mL detergent solution and then read at 570 nm (Molecular Devices SpectraMax 384 plus). Results were reported as medians ($n = 4$) with 25th and 75th percentiles of the measured absorbance normalized to the absorbance of nontreated control cells (% normalized cell viability = 100 × absorbance for cells treated with a sample/absorbance for nontreated cells).

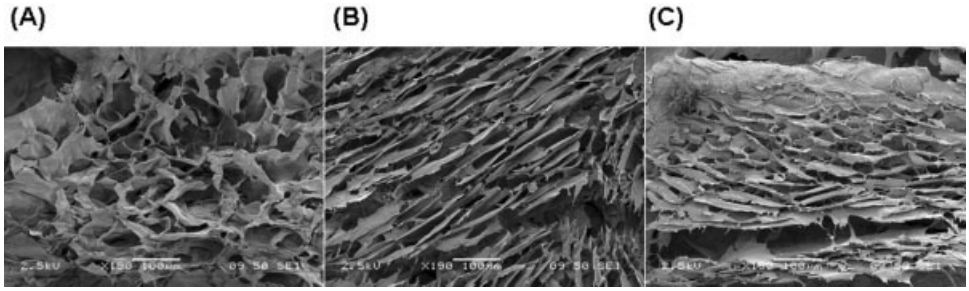

Figure 1. Scanning electron micrographs of (A) UV-cross-linked Az-chitosan, (B) noncross-linked Az-chitosan, and (C) chitosan. Scale bars = 100 μm.

Cytokine measurement

Mouse peritoneal macrophages were seeded in 24-well plates at a density of 150,000 cells per well in 1 mL DMEM. After overnight incubation, 100 μL of the chitosan or Az-chitosan solution in saline was added to each well to bring the final chitosan concentration in culture media to 0.2 or 2 mg/mL. Cells were also cultured on a 100 μL Az-chitosan gel (the same amount of chitosan per well as added with the solution) UV-cross-linked prior to addition of cells. Saline or lipopolysaccharide solution (100 μL; final concentration in the culture medium: 0.5 μg/mL) was added in lieu of chitosan solutions as a negative and positive control, respectively. After a 36-h incubation, the culture media were centrifuged at 2000 rpm for 3 min to separate supernatants. Concentrations of mouse TNF-α and macrophage inflammatory protein-2 (MIP-2) in the supernatants were determined by enzyme immunometric assay kits (TNF-α, TiterZyme, Assay Designs, Ann Arbor, MI; MIP-2, Quantikine, R&D systems, Minneapolis, MN). The minimum detectable dose of each cytokine is as follows: TNF-α, 13.0 pg/mL; MIP-2, 1.5 pg/mL.

In vivo biocompatibility

Animals were cared for in compliance with protocols approved by the Massachusetts Institute of Technology Committee on Animal Care, in conformity with the NIH guidelines for the care and use of laboratory animals (NIH publication no. 85–23, revised 1985). Female albino rabbits (*Oryctolagus cuniculus*; New Zealand White, Covance, Hazleton, PA) (2–3 kg) were used as model animals. Anesthesia was induced using Ketamine (35 mg/kg i.m.) and Xylazine (5 mg/kg i.m.); maintenance was achieved using 1–3% isoflurane in balance oxygen administered via endotracheal tube. Aseptic technique was used throughout. The animals were provided with 10 mL/kg/hour of lactated Ringer's solution throughout the surgery and the vital signs were monitored continuously. A 10 cm long midline incision was made along the linea alba on the abdominal wall, and the peritoneum was opened. In the first group, 5 mL of 20 mg/mL chitosan solution was instilled into the peritoneal cavity using a 10-mL syringe ($n = 4$). In the second group, 5 mL of Az-chitosan solution (20 mg/mL) was placed on the antimesenteric side of the mid-cecum, and the gel precursor solution was converted to the gel by longwave UV irradiation for 1 min per 1 mL ($n = 4$). A single animal received UV irradiation of the peritoneum for 5 min alone. After the treatments, the peritoneum and abdominal wall were closed with 2–0 ethilon and 3–0 dexon, respectively. The skin was closed with 3–0 ethilon. Animals were awakened and allowed to have food and water *ad libitum*. Buprenorphine (0.02–0.03 mg/kg s.c.) was administered every 8 hours for 48 hours postsurgery. In each group of four, three animals were euthanized with sodium pentobarbital after 2 weeks, one after 4 weeks. The animal that only received UV irradiation was euthanized with sodium pentobarbital after 2 weeks. Tissues were fixed in 10% formalin, embedded in paraffin, sectioned, and stained with hematoxylin & eosin (H&E) or Safranin-O/Fast Green & iron–hematoxylin as described.[26]

Statistical analysis

As the numeric data did not follow a normal distribution, they were expressed as medians with 25th and 75th percentiles. Statistical inferences were made using Mann-Whitney *U* tests and Kruskal-Wallis tests using SPSS software (Chicago, IL). A p-value <0.05 on a 2-tailed test was considered statistically significant.

RESULTS

Chitosan characterization

^{1}H NMR and UV spectra confirmed that chitosan was successfully modified with 4-azidobenzoic acid to form Az-chitosan. From ^{1}H NMR spectra, it was estimated that 12% of the amino groups in chitosan were conjugated with azidobenzoic acid: chitosan 6H's (δ 2–4 ppm) and aromatic 4H's (δ 7–8 ppm). Scanning electron microscope observation showed that the cross-linked Az-chitosan hydrogel had continuous circular or polygonal pores, typical of cross-linked hydrogels,[27] whereas matrices of both noncross-linked

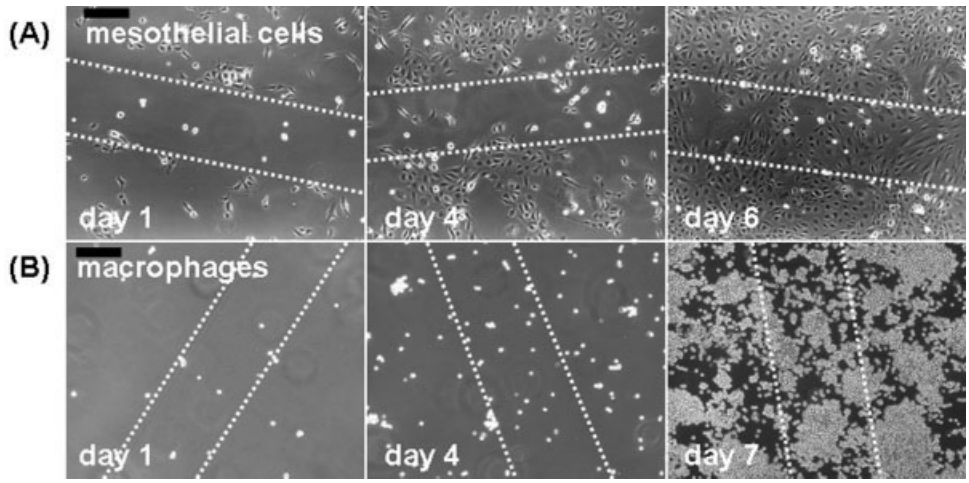

Figure 2. Light micrographs of (A) human mesothelial cells and (B) mouse peritoneal macrophages grown around stripes of UV-cross-linked Az-chitosan. Dotted lines indicate the edges of stripes. Scale bars = 200 μm.

Az-chitosan and chitosan consisted of multiple layers of sheets (Fig. 1).

Cell–gel interaction

After one day, most mesothelial cells on the uncoated part of the culture dish had attached and started to proliferate. In contrast, few cells attached to the part coated with cross-linked Az-chitosan gel, and those maintained a rounded morphology different from the spread-out shapes of adherent cells found on the uncoated part of the culture dish [Fig. 2(A)]. Cells eventually migrated onto the Az-chitosan gel over the next few days. Attachment and growth of the cells was very limited among those seeded in a well that was completely coated with the chitosan gel for 7 days (data not shown). The cross-linked Az-chitosan gels did not appear to have an impact on the distribution of peritoneal macrophages [Fig. 2(B)].

Cytotoxicity test

Prior to *in vivo* testing, we sought to assess the cytotoxicity of the materials and their components *in vitro* using cultures of the predominant cell types in the peritoneum: mesothelial cells and peritoneal macrophages. The MTT assay showed no reduction in the viability of human mesothelial cells when exposed to 0.2 mg/mL of chitosan or Az-chitosan, and statistically significant ($p < 0.05$) but relatively minor reductions with 2 mg/mL chitosan solutions [Fig. 3(A)]. Concentration had a larger effect on cell viability than chemical modification. When the mesothelial cells were grown in the wells coated with Az-chitosan gels,

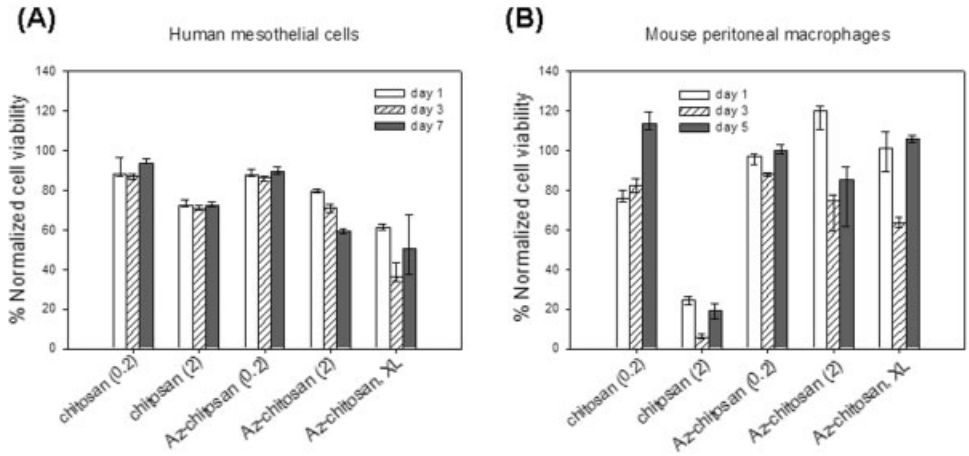

Figure 3. Cell viability in the presence of chitosan, Az-chitosan, and UV-cross-linked (XL) Az-chitosan (20 mg/mL). Data are medians with 25th and 75th percentiles ($n = 4$).

TABLE I
Effects of Chitosans Intraperitoneally Applied to Rabbits

Materials	UV Treatment	Adhesions	Weight Loss (%)
Chitosan (20 mg/mL, 5 mL)	No	4/4	11.8 ± 4.3
Az-chitosan (20 mg/mL 5 mL)	1 min/1 mL (=5 min)	4/4	16.5 ± 8.1
No chitosan	5 min	0/1	4.8

51.8% cells survived after a 7 day-incubation. Peritoneal macrophages showed little decrease in viability except for those in the 2 mg/mL chitosan solution.

In vivo biocompatibity

Laparotomies were performed, and the surface of the cecum was treated with chitosan solution ($n = 4$), *in situ* UV-cross-linked Az-chitosan ($n = 4$), or an equal UV exposure without chitosan ($n = 1$), as detailed in Methods. Two weeks (3 animals per group) or four weeks (1 animal per group) later, animals were euthanized and a necropsy was performed. The animals did not show any sign of distress in the interval between surgery and sacrifice, but lost 11.8 ± 4.3% (chitosan), 16.5 ± 8.1% (Az-chitosan), and 4.8% (UV treated control) of body weight (Table I). All animals treated with chitosans or cross-linked Az-chitosan developed adhesions over large areas of the peritoneal cavity, with numerous yellowish nodular structures dispersed throughout the adhesion (Fig. 4). Separation of the adhesions required sharp dissection. The peritoneum of the single animal that received only UV irradiation appeared to be normal. On light microscopy of hematoxylin–eosin-stained sections, the nodules appeared to be granulomatous, consisting of a core of homogeneous eosinophilic material with invaginations of inflammatory cells [Fig. 5(A)], particularly neutrophils [Fig. 5(B)]. On the periphery were numerous macrophages and lymphocytes enclosed by a fibrous capsule with fibroblasts [Fig. 5(C)]. Staining with Safranin-O/Fast Green & iron–hematoxylin confirmed that the eosiniphilic material was chitosan based [Fig. 5(D)].

Cytokines

To help explain the occurrence of marked adhesions *in vivo*, we investigated the possibility that these substances were causing adhesion development by eliciting the release of cyto- or chemokines, which in turn activate or recruit inflammatory cells, potentially leading to tissue injury. In particular, we studied whether the materials tested here induced peritoneal macrophages to secrete TNF-α and MIP-2. TNF-α is a proinflammatory cytokine, which stimulates endothelial cells and macrophages, leading to a variety of inflammatory events.[28] MIP-2 is a murine functional homologue of interleukin-8,[29] a chemokine which attracts and activates neutrophils. All chitosan formulations tested induced statistically significant elevations in MIP-2 and TNF-α from peritoneal macrophages (except that TNF-α was significantly reduced by 2 mg/mL chitosan). The levels of MIP-2 and TNF-α induced by cross-linked Az-chitosan were 20 and 55% respectively (both $p < 0.05$) of those induced by an Az-chitosan solution containing the same amount of Az-chitosan (2 mg/mL), and were 76% higher ($p =$

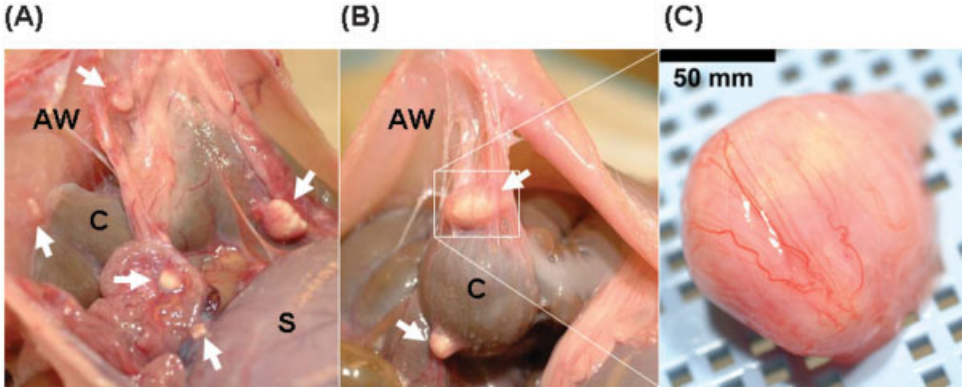

Figure 4. Adhesions from intraperitoneally applied chitosan solution: (A) after 2 weeks, (B) after 4 weeks, and (C) close-up of the nodule from (B). Note the vascularity of the lesion. AW, abdominal wall; C, cecum; S, stomach; arrows, nodules. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

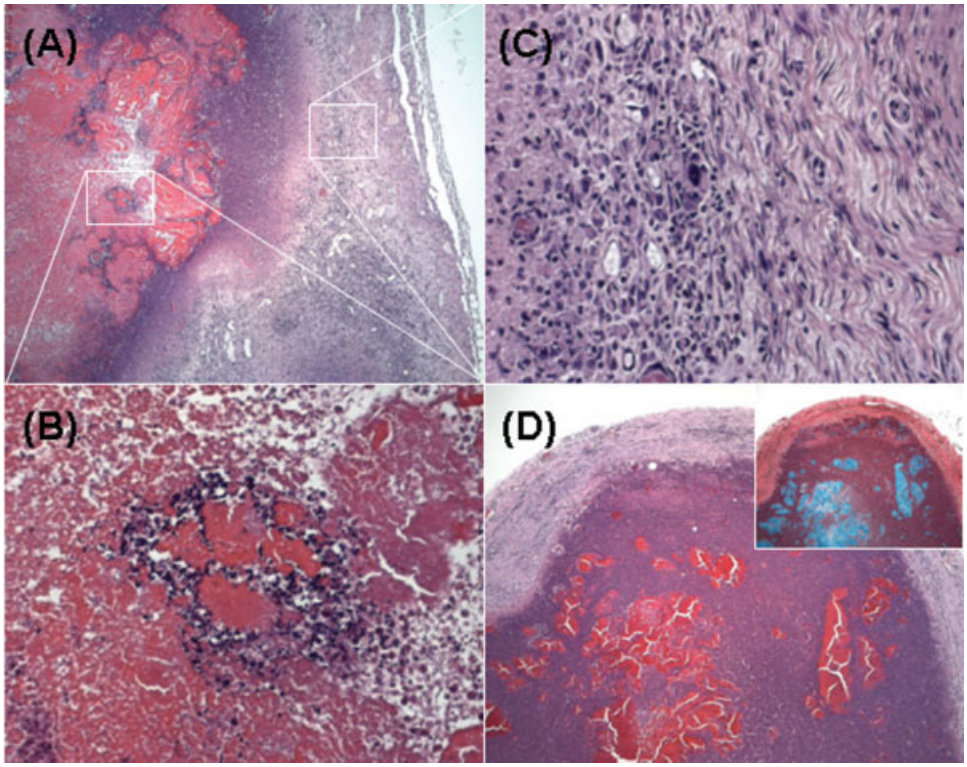

Figure 5. Tissue reaction to chitosan 2 weeks after treatment. (A) Photomicrograph of hematoxylin–eosin-stained section of nodule (50×). (B) Close up from panel A (400×), at center of lesion. The homogeneous eosinophilic material is infiltrated with cells (which on higher-powered views are neutrophils). (C) Close up from panel A (400×), at periphery of lesion (the left border in toward the lesion center) showing macrophages, lymphocytes and further out fibroblasts forming a capsule. (D) Photomicrograph of hematoxylin–eosin-stained section of nodule. The inset shows the same section stained with Safranin-O/Fast Green & iron–hematoxylin,[26] where chitosan appears bright green. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

0.029) and 22% lower (p = not significant) than from 0.2 mg/mL Az-chitosan solution. This result indicates that the cytokine induction may be mediated by the soluble leach-out of the Az-chitosan gel (Fig. 6).

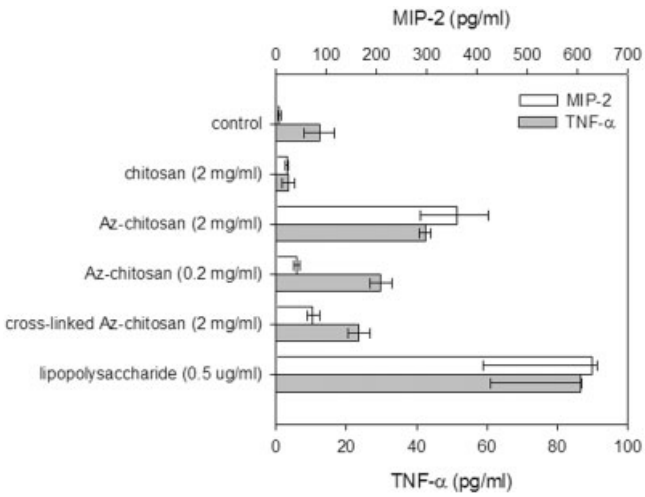

Figure 6. MIP-2 and TNF-α released from mouse peritoneal macrophages incubated with chitosan, Az-chitosan, and lipopolysaccharide. Data are medians with 25th and 75th percentiles (n = 4).

DISCUSSION

Chitosan has widely been investigated for biomedical applications in the past decade. The ever-increasing popularity of chitosan stems from its degradability in the presence of lysozyme, an enzyme prevalent in the human body[30,31] and the biocompatibility demonstrated in a few examples.[17,32] Claims have been made regarding the potential applicability of chitosan in the peritoneum, despite reports suggesting proinflammatory potential (albeit following repeated injection).[23]

We have explored the use of chitosan and its UV-cross-linkable derivative (Az-chitosan) as potential postsurgical adhesion barriers or drug delivery systems using relevant *in vitro* and *in vivo* models. We found that, at least in a rabbit laparotomy model, cross-linked Az-chitosan caused an intense granulomatous reaction with adhesion formation in all animals tested. This response also occurred with unmodified chitosan, but not with UV irradiation, suggesting that it is the material itself that is responsible for the reaction rather than UV irradiation or the process of cross-linking, although we cannot rule out the possibility that the reactive intermediate produced by irra-

diation of the gel precursor contributed to tissue injury. (Conversely, cross-linking did not appear to have a protective effect in mitigating chitosan's proadhesion effects.) Also, since only 12% of amino groups in chitosan were modified to produce Az-chitosan, we cannot exclude the possibility that the tissue reaction to cross-linked Az-chitosan gels is due to the release of unmodified or modified chitosan.

The extensive *in vivo* tissue reaction to the chitosans was surprising, given the *in vitro* observation showing that the cross-linked Az-chitosan gel itself did not preferentially attract macrophages in culture [Fig. 2(B)], and initially repelled mesothelial cells [Fig. 2(A)]. However, the Az-chitosans did induce high levels of proinflammatory cyto- and chemokines such as TNF-α and MIP-2 (Fig. 6), which suggests that soluble mediators could play a role in inducing the widespread inflammatory reactions, with subsequent adhesion formation. It is not clear that the same was true for the unmodified chitosan, where there was minimal effect on cytokine induction. In this case, the significant inhibition of macrophage viability in the presence of unmodified chitosan [Fig. 3(B)] may account for the low level of mediator secretion. Furthermore, it is possible that the cytotoxic effects of chitosan (i.e. dead cells) exert a chemotactic effect. We also note that in the absence of pharmacokinetic data regarding chitosan clearance in the peritoneum, we cannot know the extent to which macrophage viability is suppressed *in vivo*.

The greater toxicity of chitosan compared to Az-chitosan in peritoneal macrophages may be related to the degree of deacetylation of chitosan (i.e. the number of primary amines). The cytotoxicity of some polyamines in phagocytic cells has been documented.[33] Az-chitosan has fewer primary amines than unmodified chitosan, as 12% of the amino groups in chitosan were conjugated to 4-azidobenzoic acid. Although it is not clear that the toxicity of chitosan was related to phagocytic capability, we note that the nonphagocytic mesothelial cells were in general less susceptible to chitosan toxicity. The relatively low viability of mesothelial cells grown on the cross-linked Az-chitosan gels [Fig. 3(A)] may be related to the finding that those gels did not support cell attachment well [Fig. 2(A)], i.e. the low measured cell viability may be a reflection of poor attachment rather than cytotoxicity. Also note that these cells were directly seeded on the cross-linked Az-chitosan gels unlike the other conditions, in which the seeded cells were allowed to grow overnight prior to addition of the samples. Therefore, the cells grown on the cross-linked Az-chitosan gels were exposed to the test material longer than the other condition by one day, which could also be one of the reasons for the relatively low viability of cells grown on the cross-linked gels.

This study highlighted some of the difficulties inherent in *in vitro* tests, which are used by many as a surrogate for biocompatibility. Neither the cell migration experiments (Fig. 2) nor the cell viability assay (Fig. 3) give much cause for concern regarding the use of chitosans in the peritoneum, aside from the low level of macrophage viability with 2 mg/mL chitosan (which could be interpreted as being protective). Much depends on which cells are tested; it may be hard to test all the important cell types involved in a complex milieu such as the *in vivo* environment. Such tests also do not reflect cell–cell interaction, particularly long-range ones mediated by soluble factors. In that regard, assays of the release of factors that could cause tissue injury (Fig. 6) may provide useful additional information.

Here we showed that intraperitoneally applied chitosans induced extensive inflammatory reactions, leading to granulomas and adhesions in the peritoneal cavity in a rabbit model. Our observation suggests that chitosan may not be biologically inert but can lead to significant inflammatory reactions through indirect chemotactic activity. Although its adhesive and even proinflammatory properties may be beneficial in some biomedical applications such as wound healing,[12] it needs to be carefully evaluated as a biomaterial for peritoneal administration.

The authors are grateful to Dr. Jianjun Cheng for the valuable discussion and help with NMR analysis, and to Evangelia Bellas and Kathryn Madden for technical support.

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