Fetal Abdominal Wall Repair with a Collagen Biomatrix in an Experimental Sheep Model for Gastroschisis

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We evaluated the regeneration of the abdominal wall using a dual-layer collagen biomatrix, and the protective effect on the bowel of fetal abdominal wall repair in a fetal sheep model for gastroschisis. In 14 fetal lambs, the abdominal wall was opened at 79 days' gestation, creating a gastroschisis. In group 1, the gastroschisis was left uncovered. In group 2, the bowel was repositioned, and the defect was closed by suturing a collagen biomatrix into the abdominal wall. A cesarean section was performed at 140 days' gestation, and macroscopic and histological evaluation was performed. In the five lambs with a gastroschisis, the eviscerated part of the bowel was coalescent, showed extensive adhesions, and was covered by fibrous peel. In group 2, the abdominal wall had closed, with a firm connection to the native abdominal wall. The biomatrix was largely degraded and replaced by connective tissue with collagen and fibroblasts, neovascularisation, and scattered muscle cells. Minor or no adhesions of the bowel and no peel formation were observed. Abdominal wall tissue replacement using a collagen biomatrix was feasible in fetal lambs, resulting in a closed abdominal wall at birth. Immediate closure of the gastroschisis strongly diminished or prevented bowel adhesions and peel formation.

Introduction

SURGICAL CLOSURE of congenital abdominal wall defects can be a complex problem for pediatric surgeons. An important group is patients with gastroschisis. Gastroschisis is an abdominal wall defect through which a large part of the bowel is herniated outside the abdominal cavity and is in direct contact with the amniotic fluid. The mortality in these children is approximately 10%, but intra-uterine growth retardation and premature birth are frequent, and serious complications such as sepsis, bowel dysfunction, bowel atresia, bowel necrosis, and subsequent short bowel syndrome can occur.[1-4] At birth, the bowel is often covered with an inflammatory fibrous peel; the bowel loops are matted together and can be congested or ischemic; and the bowel is thickened, inflamed, and edematous. Constriction of the bowel at the site of the abdominal wall defect and the toxic effect of the amniotic fluid cause this damage to the bowel.[5,6] The damage to the bowel seems to originate during the last trimester of pregnancy. At that time, the bowel is growing, and compression at the abdominal wall defect will occur.

Furthermore, the composition of the amniotic fluid is changing because of improving kidney function and the loss of gastrointestinal waste products.[7-11] The surgical repair of these defects can be complicated using difficult primary closure because of the hypoplastic abdominal cavity and the enlarged volume of the bowel because of edema and peel formation. An increase in intra-abdominal pressure can occur after repositioning the bowel in the abdominal cavity, causing respiratory problems and compromised venous blood flow. In these cases, a gradual closure with a spring-loaded silo is chosen.[12] In some cases, even prosthetic materials are needed to close the abdominal wall defects or the fascia defects. These materials may also cause complications, such as wound infection, bowel fistula, erosion into abdominal viscera, lack of fixation, mesh extrusion, and adhesion formation.[13] Furthermore, patch dehiscence may occur because the material does not grow with the child.[14] Gastroschisis can be detected in early pregnancy,[15] which offers the opportunity to salvage the bowel tissue using fetal therapy. Tissue-engineered constructs could be a solution for the operative closure of these defects.

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The purpose of this study was to repair the full-thickness defect in the abdominal wall in fetal lambs with a surgically created gastroschisis using a molecularly defined acellular collagen biomatrix to induce regeneration of abdominal wall tissue, and to protect the bowel. We evaluated tissue regeneration in the biomatrix, the inflammatory response, the protective effect of abdominal wall closure with the biomatrix on bowel tissue, adhesions of the biomatrix to underlying tissue, and the presence of hernias.

Materials and Methods

The Ethical Committee on Animal Research of the Radboud University Nijmegen Medical Centre approved this study under protocol number RUDEC 2003-96.

Preparation of collagen biomatrices

The molecularly defined, biocompatible, biodegradable dual-layer collagen biomatrices were made from insoluble highly purified type I collagen from bovine Achilles tendon.[16] The biomatrix consisted of a porous layer and a dense film layer. A 0.8% (w/v) type I collagen suspension was shaken overnight in 0.25 M acetic acid at 4°C and homogenized on ice using a Potter-Elvehjem homogeniser. Air bubbles were removed by centrifuging at 250 g for 10 min at 4°C. The suspension was slowly poured into a plastic mold (4 mL per Ø 32 mm), and air-dried for 3 days at 22°C to prepare a flat film layer. These films were incubated for 60 min in 4 mL 0.25 M acetic acid. Acetic acid was then removed, and a suspension of 4 mL 0.8% type I collagen in 0.25 M acetic acid was poured on top of the films, quickly frozen at −80°C, and lyophilized in a Zirbus lyophiliser (Bad Grund, Germany). Scaffolds were cross-linked using 33 mM 1-ethyl-3-(3-dimethyl aminopropyl)carbodiimide and 6 mM N-hydroxysuccinimide in 50 mM 2-morpholinoethane sulphonic acid pH 5.0 containing 40% ethanol (5 mL per Ø 32 mm) for 4 h at 22°C. Scaffolds were then washed with 0.1 M disodium hydrogen orthophosphate, 1 M sodium chloride (NaCl), 2 M NaCl, and MilliQ water, frozen again in ethanol/carbon dioxide, and lyophilized.[17,18] Biomatrix morphology was assessed using scanning electron microscopy (Fig. 1).[17,18] The porous layer had interconnective pores, and the mean average pore size of the top side of the porous layer was $106 \pm 22\,\mu m$ and of the cross-section was $123 \pm 34\,\mu m$ (average of 100 pores of three individually prepared biomatrices). The porous layer and film layer had an average diameter of 1.5 mm and 2 to 3 μm respectively. Cross-linking was verified according to its amine group content, and 48% of the amine groups were used in the crosslinking process.[17] Before implantation, the matrices were washed in 70% (v/v) ethanol and sterile phosphate buffered saline.

Surgical procedures

Fourteen pregnant sheep (Dutch Texels breed) were operated on at 79 days' gestation (full term 140–147 days). An intravenous injection of 30 mg/kg pentobarbital and 1 mL atropine was used for anesthesia and, following endotracheal intubation, was maintained with 2% isoflurane and oxygen/air ventilation at a respiration rate of 16 breaths per minute. The uterus was exteriorized through a midline abdominal incision. A hysterotomy was performed, and the lower part of the fetal body was exposed. In case of twin or
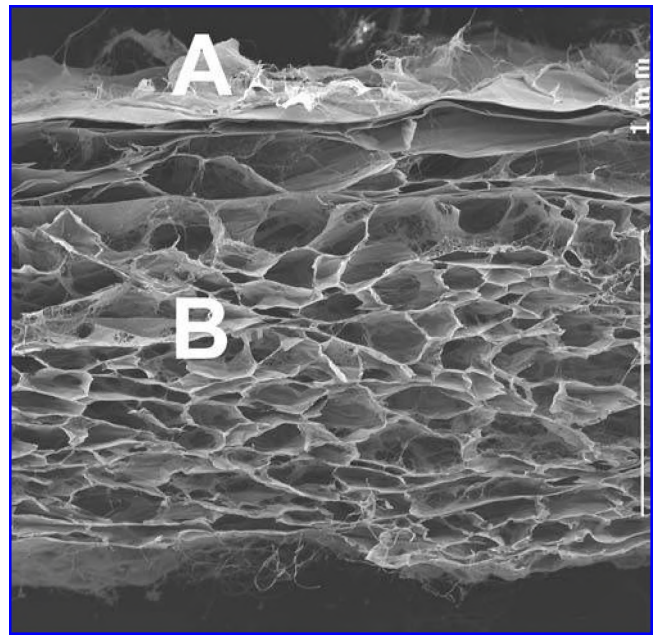

FIG. 1. Dual-layer collagen biomatrix (scanning electron microscopy). (A) Film layer of biomatrix; (B) porous layer of biomatrix.

triplet pregnancy, only one fetus was operated on to avoid additional risk of complications.

The animals were divided into two groups. In group 1, consisting of five fetuses (two male, three female), a gastroschisis was surgically created. An incision of 2.5 cm was made in the left lower quadrant of the abdominal wall of the fetuses, through skin and fascia, resulting in a full-thickness abdominal-wall defect of approximately 2.5×2 cm. The defect was left-sided to avoid injury to the liver by surgical manipulation. Subsequently, the bowel was exposed and gently extruded from the abdominal cavity (Fig. 2A). The lesion was left uncovered, leaving the bowel exposed to the amniotic fluid.

In group 2, consisting of nine fetuses (seven male, two female), a gastroschisis was created as in group 1. Subsequently, the bowel was gently manipulated back into the abdominal cavity, and the dual-layer collagen biomatrix, measuring approximately 2.5×2 cm was placed into this defect (film layer at the luminal site). The biomatrix was sutured in the abdominal wall using 6-0 poliglecaprone (Monocryl, Ethicon, Inc., Sommerville, NJ) interrupted sutures (Fig. 2B). Four 6-0 polypropylene (Prolene, Ethicon, Inc.) marking sutures were placed around the biomatrix for future reference.

After the surgical procedure, the fetus was returned to the uterus and amniotic fluid volume was restored using warm sterile saline together with amoxycilline 250 mg. The uterus was closed in two layers using a 2-0 polyglactin (Vicryl, Ethicon, Inc.) running suture. Sodium-penicillin (1,000,000 IU) was instilled into the intra-abdominal space, and the maternal laparotomy was closed in two layers using 1 polyglactin interrupted sutures. Depomycine (20 mg/kg, subcutaneous) was initiated preoperatively and maintained postoperatively for 3 days.

At 140 days' gestation, 61 days after surgery, the lambs were delivered by caesarean section under local anaesthesia
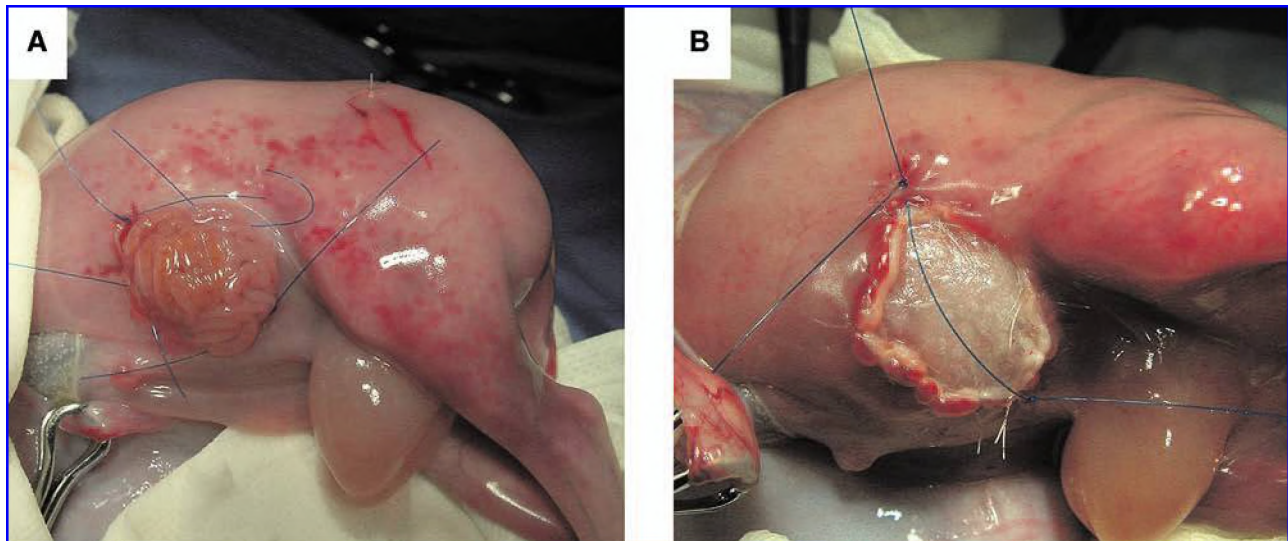
FIG. 2. Fetal lambs operated on at 79 days' gestation. **(A)** Surgically created gastroschisis. **(B)** Surgically created abdominal wall defect closed with collagen biomatrix.

with 20 to 30 mL Lidocaine 2%, administered subcutaneously and intramuscularly.

Neonatal outcome and evaluation

After birth, the macroscopic appearance of the bowel was observed and photographed in group 1, and the size of the defect was measured. The replaced part of the abdominal wall at the place of the incorporated biomatrix in group 2 was macroscopically observed, palpated, photographed, and measured. Subsequently, the lambs were sacrificed using medetomidine (0.5 mg intramuscular) and pentobarbital (60 mg/kg intracardial). Afterward, the abdominal wall of the lambs from group 1 was opened, and the bowel was taken out from pylorus to rectum, with a small part of the adhering abdominal wall. The intra- and extra-abdominal part of the bowel was evaluated for adhesions and fibrous peel.

The abdominal wall of the lambs from group 2 was opened with broad margins around the site of the former biomatrix. Intra-abdominal adhesions were observed and photographed. The abdominal wall and the bowel, from pylorus to rectum, were taken out.

Histological staining

From the lambs of group 1, tissue samples were taken from the bowel situated outside of the abdominal cavity and from bowel inside the abdominal cavity. In group 2, samples from the replacing tissue at the site of the implanted collagen biomatrix and tissue samples of the bowel lying
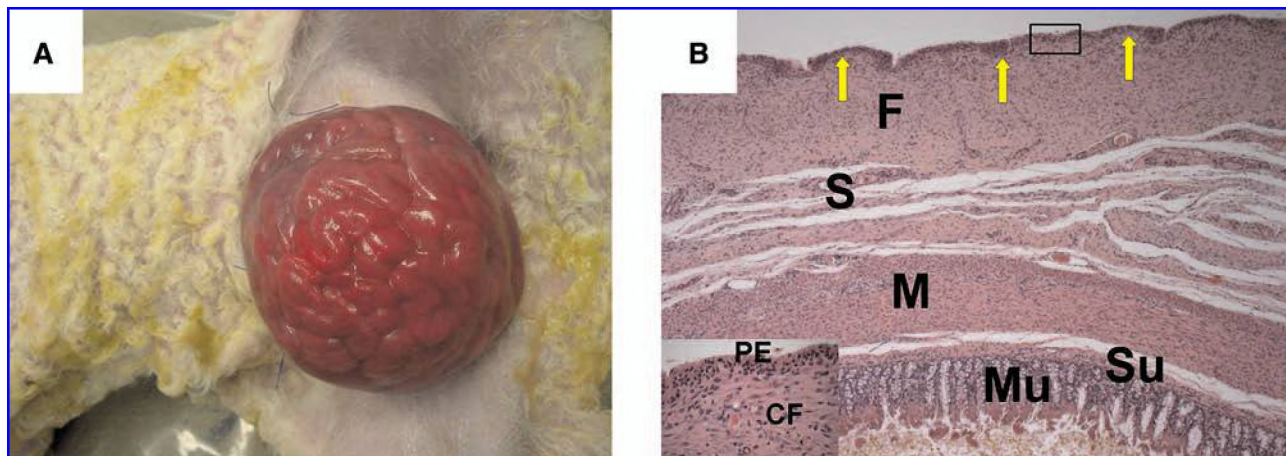

FIG. 3. **(A)** Macroscopic aspect of the surgically created gastroschisis (group 1), after birth, showing coalescent bowel loops covered with fibrous peel. **(B)** Histological picture of the bowel wall of a lamb from group 1, after birth, showing the fibrous peel. Arrows = pseudoepithelial layer of mesenchymal cells; F, fibrous peel; S, subserosal layer; M, intestinal muscularis; Su, submucosa; Mu, mucosa (hematolyxlin and eosin (H&E) staining, original magnification ×50). (Magnification of rectangle in inset). Inset: PE, pseudoepithelial layer of mesenchymal cell; CF, collagen and fibroblasts (H&E staining; ×40).

underneath the site of the implanted collagen biomatrix were taken. Tissue samples of normal bowel of five lambs that had undergone a fetal operation in another study served as controls.

The tissue samples were fixated in 4% buffered formalin and paraffin embedded for routine histological processing. Sections (4 μm) were cut and stained with hematoxylin and eosin and Masson's trichrome. The intestinal tissue of the lambs was examined for changes in the mucosal, submucosal, muscle, and serosal layers and peel and adhesion formation. Two bowel samples were used from each fetus to assess the thickness of the intestinal muscularis and the serosal peel layer in three random fields with an ocular micrometer at magnification ×100. The specimens, which included the site of the implanted collagen biomatrix in the abdominal wall, were examined for evidence of epithelialization, smooth muscle cell growth, neovascularization, and degradation of the biomatrix. In addition, the thickness of the replacing tissue was measured using the ocular micrometer. Immunohistochemical staining was performed using desmin for staining of muscle cells and S-100 staining to visualize nerve fibres.

Data analysis

Data analysis of the bowel measurements was performed using SPSS 12.0 for Windows (SPSS, Inc., Chicago, IL) and expressed as means ± standard deviations. Statistical analysis was performed using one-way analysis of variance. $P < 0.05$ was considered statistically significant.

Results

Eleven of the 14 operated fetuses (79%) were born alive. Two fetal deaths occurred in group 2 without a clear reason; the lambs were found macerated at the caesarean section. One ewe was euthanized because of an infection in the fascia of the abdominal wall, resulting in uncorrectable fascia dehiscence. No further maternal deaths occurred.

The five control lambs showed no macroscopic or histological bowel changes. At histological examination, the serosa measured 0.02 ± 0.01 mm and the intestinal muscularis 0.08 ± 0.03 mm.

After birth, four lambs of group 1 showed eviscerated bowel covered with a fibrous peel; in two lambs, a thick layer was present, and in two lambs a clearly thinner layer of fibrous peel was visible (Fig. 3A). In one lamb, the bowel had spontaneously repositioned into the abdominal cavity, and the abdominal wall had closed with a small scar. The bowel of this lamb was enveloped in a sac, with the same appearance as the fibrous peel. The eviscerated bowel package of the other four lambs measured approximately 7×5×5 cm in size, and the size of the abdominal wall defect was 5 cm. The eviscerated part of the bowel was coalescent and showed extensive, inseparable adhesions. The intra-abdominal part of the bowel also showed adhesions, although of a much lesser extent than the eviscerated bowel. No atresia or other bowel abnormalities were seen.

Histological examination of the intestines of the gastroschisis lambs (group 1) showed that the serosa of the four lambs with the eviscerated bowel was covered with fibrous peel. In two lambs, the fibrous peel was thick (mean diameter 1.37 ± 0.36 mm and 2.64 ± 0.91 mm). The peel consisted of deposited fibrin and degenerated granulocytes, granula-

tion tissue with chronic inflammation (focal foreign body giant cells around hair remnants, plasma cells, lymphocytes, and histiocytes), fibrosis, and focal hemosiderin pigment. In two lambs, the fibrous peel was thinner (0.75 ± 0.23 mm) and without inflammation. In these lambs, a pseudo-epithelial mesenchymal layer of cells, which seemed to protect the bowel tissue against the amniotic fluid, covered the peel (Fig. 3B). No edema, venous dilatation, lymphatic dilatation, or signs of ischemia were seen in the bowel tissue. The mucosa appeared normal, with normal villi, and the submucosa was normal, without collagen deposits. The intestinal muscularis showed some thickening (0.14 ± 0.05 mm) with collagen deposition; normal ganglion cells were seen. The bowel of the lamb with the spontaneously closed defect showed no abnormalities, with a normal serosal layer (mean diameter 0.02 mm).

The six surviving lambs of group 2 showed a closed abdominal wall; the replacing tissue was visible between the marking sutures in the skin after shaving, measuring approximately 3.2 ± 0.8 cm in diameter (Fig. 4A). The replacing tissue was of strong consistency; in one lamb, a small herniation in the abdominal wall of 1.5 cm was palpable under the skin, and in the other lambs, no herniations were palpable. In the center, a hyperkeratotic area was seen in five of six lambs, and the surrounding tissue formed a ridged configuration. Underneath this regenerated tissue, the bowel had a normal appearance, no adhesions between bowel loops were visible, and only some minor adhesions between the bowel and the abdominal wall existed (Fig. 4B). The bowel tissue in these lambs appeared normal on histological examination. No peel formation was found; the serosal layer (0.02 ± 0.01 mm) and the intestinal muscularis (0.08 ± 0.03 mm) were of normal thickness and significantly thinner (both $p < 0.001$) than in gastroschisis lambs. The submucosa and mucosa appeared normal (Fig. 4C). Histological examination of the abdominal wall showed tissue replacement throughout the entire biomatrix in all lambs. Connective tissue, with collagen and fibroblasts, was largely replacing the biomatrix. There was a firm connection with the adjacent skin, subcutaneous tissue, and muscle of the native abdominal wall. The replacing tissue was thinner than the native abdominal wall, with the native abdominal wall measuring 6.2 ± 1.4 cm and the replacing tissue 3.6 ± 1.4 cm (Fig. 5A, B, D). On the outside, the entire replacing tissue was covered with skin tissue in all lambs. The skin tissue was more mature at the edges of the newly formed tissue, with epithelialization and adnexal differenattiation, including sebaceous glands and hair follicles, than at the center of the tissue (Fig. 5A). Tissue replacement occurred from the borders of the regenerated tissue. Hyperkeratinization was visible at the center of this tissue in five of six lambs (Fig. 5B). Good neovascularization was seen throughout the entire replacing tissue, at the borders as well as the center of this tissue (Fig. 5A–C). The angiogenesis seemed to originate from the peritoneum and the edges of the native abdominal wall. Only minor inflammatory reaction was seen, without a foreign body reaction. The collagen biomatrix was largely degraded, except for the less-porous film layer, which was still visible at the inside of the abdominal wall (Fig. 5A–C). A small number of scattered muscle cells were visible in the replacement tissue, surrounded by myofibroblasts, but no muscle bundles were

FIG. 4. (**A**) Abdominal wall defect closed using a collagen biomatrix (group 2), macroscopic aspect after birth, showing a closed abdominal wall with regenerated skin tissue. (**B**) Minor adhesions visible (arrows) in a lamb from group 2. (**C**) Histological picture of the bowel of a lamb from group 2, showing no abnormalities. Se, serosal layer; M, intestinal muscularis; Mu, mucosa (hematolyxlin and eosin staining; original magnification ×50).
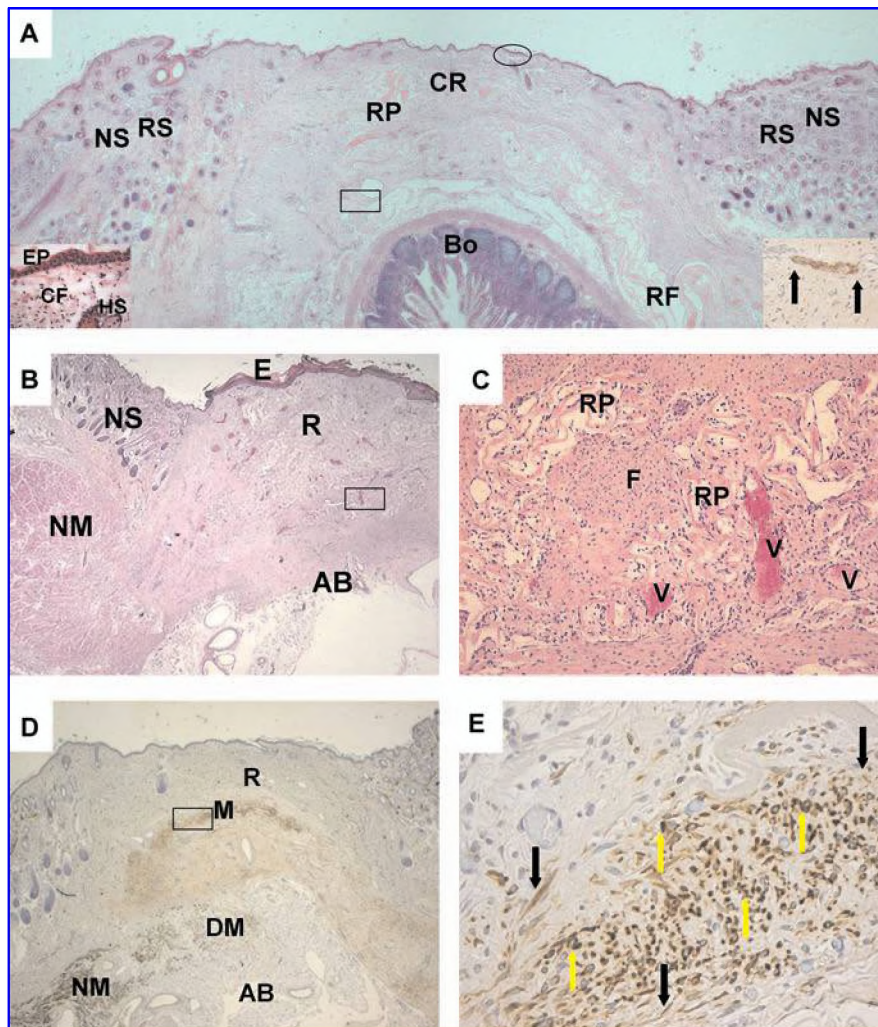

FIG. 5. Histological imges of the replacing tissue at the side of the abdominal wall defect closed using a collagen biomatrix (group 2). (**A**) Overview of replacement tissue showing that regenerated skin and connective tissue replaced the biomatrix. The regenerated skin tissue (RS) (=between RS) at the border was more mature, with hair follicles and sebaceous glands, than at the center (CR). The replacement tissue was thinner than the native abdominal wall. Remnants of the film layer (RF) and porous layer (RP) were visible. NS, native skin; Bo, bowel. (Hematolyxlin and eosin (H&E) staining; original magnification ×12.5). Inset on the left: magnification of the ellipse at the overview, showing epidermis (EP), collagen and fibroblasts (CF), and hair follicle and sebaceous gland (HS) (H&E staining; ×40). Inset on the right: nerve fiber in regenerated tissue (arrows) (S-100 staining; ×200), located at the rectangle in overview. (**B**) Overview of regenerated tissue, showing the border between native skin (NS) and native muscle (NM) and the replacement tissue (R). The skin is covered with a hyperplastic epidermis with hyperkeratinization (E). AB, abdominal site. (H&E staining; original magnification ×25). (**C**) Connective tissue formation, with fibroblasts and collagen (F) and mature vessels (V). RP, remnant of porous layer biomatrix. (Magnification of rectangle in Fig. 5B). (H&E staining; ×100). (**D**) Replacement tissue (R) with scarce smooth muscle cell formation (M). The native muscle (NM) was partially degenerated (DM). AB, abdominal site. (Desmin staining; ×12.5). (**E**) Magnification of smooth muscle cells; yellow arrows = rounded striated muscle cells; black arrows = spindle-shaped myofibroblasts. (Magnification of rectangle in fig. 5D). (Desmin staining; ×200).

seen (Fig. 5D, E). Only few nerve fibers could be found in this tissue (Fig. 5A).

Discussion

Fetal therapy has been applied for several congenital anomalies in experimental settings. This was first performed in life-threatening anomalies, but nowadays it is also used for the treatment of non-fatal anomalies, as in gastroschisis.[2] The survival rate in gastroschisis patients is commonly greater than 90%, but various problems may arise after birth, and some report a postnatal complication rate of 79%.[4] The damage to the bowel is assumed to occur during late pregnancy, due to constriction of the bowel at the abdominal wall defect and exposure to the amniotic fluid.[5] Early detection of gastroschisis during pregnancy is possible using routine ultrasound screening and offers the opportunity for early treatment during the fetal period, to protect the bowel tissue against further secondary injury. Till et al. successfully repaired the abdominal wall of rabbit fetuses with a surgically created gastroschisis using operative closure, but the follow-up period was negligible.[19] Tissue-engineering techniques have been used to close abdominal wall defects in adult animals. In these studies, full- or partial-thickness abdominal wall defects were surgically created, and scaffolds of extracellular matrix were used to close these defects, resulting in firm connective tissue formation, degradation of the scaffold, and some reported regeneration of skeletal muscle.[14,20–25] Acellular scaffolds have also been used experimentally in small numbers of humans with large abdominal wall defects that were inappropriate for primary closure.[26,27] In previous work, we used a collagen biomatrix to cover a surgically created neural tube defect in fetal lambs.[28,29]

In the present study, we used the fetal lamb model for surgical creation of gastroschisis. The lambs in which the bowel was left eviscerated out of the abdomen showed macroscopic and histological similarity with gastroschisis in humans. Extensive adhesions and peel formation were seen in these lambs.

In one lamb, the bowel had repositioned in the abdomen, and the abdominal wall had closed. Spontaneous intrauterine closure of the defect in humans has been reported, accompanied by intestinal involution resulting in intestinal atresia.[15,30] In our animal model, the defect in the abdominal wall was larger than the defects in humans, which may explain the spontaneous repositioning of the bowel in one lamb, and the absence of signs of bowel constriction at time of birth.

In the other group, the full-thickness abdominal wall defect was immediately repaired using a collagen biomatrix, and the regeneration of abdominal wall tissue and possible changes of the bowel were studied. We used a molecularly defined, biocompatible, biodegradable dual-layer biomatrix of highly purified type I collagen, which is a modification of the biomatrix previously used for fetal closure of spina bifida, to repair the abdominal wall.[28,29,31] The modification consisted of adding an additional thin layer of collagen with less porosity and higher strength capabilities, to increase the total tensile strength of the biomatrix. In all lambs, the defect in the abdominal wall had closed after birth. Histological examination showed that the porous layer of the biomatrix was largely degraded, but the film layer resided. Connective

tissue and skin formation replaced the porous layer of the collagen biomatrix. Good neovascularization occurred throughout the entire replacement tissue. No inflammatory response to the biomatrix was visible. It is likely that this was due to the use of highly purified collagen, instead of decellularized tissue that is often used in other animal studies. The tissue appeared to be firm and was well incorporated into the native abdominal wall tissue. In one lamb, a small herniation was palpable; in the other five lambs, no herniations occurred. Only a small group of muscle cells was seen in the repaired area, surrounded by spindle-shaped myofibroblasts. Some groups have reported varying degrees of muscle regeneration,[21–25] but the follow-up period of 61 days in our study could be too short; the tissue would probably have developed further during the postnatal period if the lambs had not been sacrificed. The bowel appeared normal on macroscopic and histological examination. Only minor adhesions between the bowel and the abdominal wall occurred with this collagen scaffold, without further complications, and no peel formation or inflammatory reaction was seen in the bowel. Immediate closure of the abdominal wall defect prevented these alterations.

In human studies in which a collagen biomatrix was used to close abdominal wall defects, the major disadvantage was the low tensile strength of the collagen biomatrix, especially when degradation of the collagen occurred.[13] By modifying the biomatrix using chemical cross-linking of the collagen, the degradability can be adjusted to make it more appropriate for abdominal wall closure, and the strength is greater.[16–18] The addition of the film layer with less porosity further improves the strength and maintains this strength for a longer period because of its lower degradability. Furthermore, improved regeneration of the muscle and fascia would also provide extra strength to the newly formed tissue. A possible strategy of improving the regeneration could be the incorporation of growth factors into the biomatrix to enhance neovascularization and ingrowth of (muscle) cells. The incorporation of autologous muscle cells also seems to improve cell infiltration and mechanical performance.[22–25,32,33] However, this technique may be inappropriate for fetal operations, because an additional operation for muscle biopsies will then be needed, increasing the risk of complications.

The use of biomaterials can be a promising tool for patients with an abdominal wall defect to regenerate the abdominal wall or cover the eviscerated bowel. Fetal therapy may have its advantages in these patients; early coverage of the bowel could prevent inflammatory changes and adhesion formation and might salvage the bowel function. Furthermore, the defect is smaller at this point, adequate neovascularization may be obtained throughout the entire biomatrix, and the incongruence of volumes between the abdominal cavity and the bowel is smaller than in neonates. Fetal wound healing has a strong potency and can even result in scarless wound healing.[34] By repairing these defects at the fetal stage, the advances of the regenerative capacity of the fetus are used. However, in our model, the regeneration process took place during gestational weeks 11 through 20, which is largely during the third trimester. Scarless wound healing and the regenerative capacity of the fetus decrease when the fetus is nearing full term, and near term it will be comparable with postnatal wound healing.[34]

Currently, the major disadvantage of fetal surgery is the risk of complications leading to premature delivery.[35,36] However, with improvements in the techniques for fetal access, it can become a promising tool for patients with this congenital anomaly in the near future.

Acknowledgments

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