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NOVEL NON-CELLULAR ADHESION AND TISSUE GRAFTING IN THE MUTABLE COLLAGENOUS TISSUE OF THE SEA CUCUMBER *PARASTICHOPUS PARVIMENSIS*

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Summary

Previous work on wound healing in holothurians (sea cucumbers) has been concerned with the relatively long-term cellular processes of wound closure and regeneration of new tissue. In this report, we characterize a short-term adhesion that is a very early step in holothurian wound healing. Dissected pieces of dermis from the sea cucumber *Parastichopus parvimensis* adhered to each other after only 2 h of contact, whether the cells in the tissues were intact or had been lysed. Lapshear tests showed that the breaking stresses of adhered tissues reached approximately 0.5 kPa after 24 h of contact. Furthermore, dermal allografts were incorporated into the live recipient individuals without any external pressures, sutures or artificial gels to keep them in place. Dislodging the grafts after 24 h of contact required shear stresses of approximately 14 kPa. It appears that the adhesive property of the dermis plays a key role in the initiation of this grafting.

Key words: mutable collagenous tissue, catch connective tissue, grafting, skin grafting, wound healing, adhesion, echinoderm, *Parastichopus parvimensis*, holothurian, invertebrate, connective tissue.

Introduction

The conspicuous wound healing ability of holothurians (sea cucumbers) has long been recognized (Torelle, 1909). Over the last 30 years, a handful of studies have examined the phenomenon more closely (Cowden, 1968; Hildemann and Dix, 1972; Menton and Eisen, 1973; Fankboner, 1978), but very little is understood about the initial stages of wound healing. These involve reapproximation (the closing of the wound and alignment of the surfaces in preparation for more gradual repair procedures such as cell migration and differentiation) and the synthesis of extracellular matrix, which is defined here as all components external to any cells. The current investigation was prompted by observations that the holothurian *Parastichopus parvimensis* accepts dermal grafts very easily and closes its wounds very rapidly, and that the wounded surfaces adhere to each other in a way that might maintain this closure. Furthermore, this adhesion occurs even between detached pieces of dermis that have had their resident cells destroyed. These observations led us to look for a non-cellular means by which this adhesion could be occurring.

Holothurian dermis is largely composed of collagenous fibers in a relatively amorphous extracellular matrix (often referred to as the ‘ground substance’) rich in proteoglycans. In this sense, its composition is similar to that of vertebrate connective tissues, and yet we witnessed adhesion between pieces of holothurian dermis in which the cells had been destroyed by freezing and thawing, while no adhesion was observed between pieces of cartilage or between dermis and cartilage. It seems, therefore, that the holothurian tissue differs in some unknown way that allows adhesion to occur. This adhesive difference might be responsible for the ease with which this tissue can be grafted, as we demonstrate in this paper. Both autografts and allografts were successfully incorporated into the dermis of most individuals (i.e. the grafts ‘took’ well). The immunological reactions to the allografts will not be a focus of this paper (the grafts were eventually rejected); they have been discussed by Hildemann *et al.* (1979).

Holothurian dermis is also considered to be a ‘mutable collagenous tissue’ (MCT). This means that its stiffness can be rapidly and reversibly altered by the animal (a phenomenon that is apparently unique to echinoderms; see Wilkie, 1996). MCTs can change from being ‘compliant’ to being ‘stiff’ within a few seconds. The mechanism for this change is still not clear, but it has been demonstrated that certain artificial conditions will reliably produce these mechanical states in the tissues. The removal of free Ca^{2+} from the extracellular space will make the MCTs compliant, while freeze-thawing will make many of them stiff (for a review, see Wilkie, 1996). In this study, we used these techniques to elicit both of these extreme mechanical states in the dermis so that we could explore the effects of MCT stiffness on MCT adhesion. We suspected that the two phenomena might be linked because they are both exceptional properties of the same extracellular matrix.

The objectives of the present study were (1) to investigate the conspicuous wound closing ability of *P. parvimensis* using

wound healing and grafting experiments, (2) to determine the role of living cells in the self-adhesive ability of the tissue, (3) to describe the junction between apposed tissues at the microscopic level, (4) to quantify the adhesion by measuring the force required to separate adhered tissues in lapshear tests, and to (5) determine whether the mechanical state of the tissue (i.e. stiff or compliant) correlates with the level of adhesion of the tissue.

Materials and methods

Previous literature has contained some ambiguity regarding the nomenclature of the tissues used in this study (Holland, 1984). Hyman (1955, p.132) describes the body wall of holothurians as consisting of a thin, external cuticle covering an epidermis, overlying a highly collagenous dermis (which constitutes the thickest part of the body wall and approximates the standard description of vertebrate dermis). This all overlies an internal layer of circular and longitudinal muscle fibers. In the present study, we used pieces of body wall from which the muscle layer had been removed, but which still possessed the thick, extracellular, collagenous feltwork covered by a thin epidermis and cuticle. We use the simplified term 'dermis' to describe each of these tissue specimens because the epidermis and cuticle account for only a very small fraction of the surface area tested.

Wounding, grafting and lapshear tests

Sea cucumbers (*Parastichopus parvimensis* Clark, 1913) of medium size (185±33.8 g wet mass, mean ± S.D., N=15) were collected with the aid of SCUBA off the coast of La Jolla, California, USA, and kept in a large (approximately 2 m^3) flow-through seawater aquarium at ambient ocean temperature (11–21 °C), where they were maintained with no mortality for at least 3 months.

Several cuts 2 cm long were made in the dermal tissues of five individuals. Cuts were made both longitudinally and circumferentially to various depths (including cuts penetrating into the coelom). In 30 additional individuals, 2 cm×1 cm×0.3 cm (depth) sections of dermis were excised. In six of these, the wounds were left exposed. In twelve others, the excised sections from each individual were replaced over the wounds (i.e. autografts). In the final twelve individuals, the same procedure was carried out except that allografts were created by switching the excised pieces between individuals. No external pressures, sutures or devices of any kind were used to hold the grafts in place. These procedures took approximately 1 min, after which the sea cucumbers were replaced into their tanks where they were allowed to resume their normal activities.

The adhesive strengths of four grafts were tested as follows. The lateral interfaces between the grafts and the host tissues were cut with a razor so that only the bottom of the graft was still in contact with the host (this was done so that the contact surfaces being sheared would be analogous to the surfaces in the lapshear tests described below). A series of small hooks embedded in a rectangular block of epoxy resin was then implanted into the graft, and it was pulled from its host by shear forces (i.e. pulled parallel to the adhered surface). The force required to do this was measured using a T10 tensometer and recorded using a chart recorder (both devices from Monsanto, Wiltshire, England). The breaking shear stress was calculated by dividing the maximum force by the area of contact between each set of tissues. This normalization of the force measurement over the area assumes a homogeneous adhesion of the surfaces, which gives the most conservative estimate of stress at any contact point.

To obtain tissue specimens for all other tests, each individual was induced to become stiff by gently drumming on its surface with a few fingers prior to cutting. This resulted in smooth, well-defined tissue specimen surfaces. The body wall was then split longitudinally along the ventral surface with a razor blade and opened up so that its inside surface was exposed. The viscera and muscle layers on the inside of the body wall were removed using forceps, leaving only the collagenous dermis. Tissue samples were always taken from the central dorsal area of the dermis, where there are no tube feet. Standard-sized tissue samples were excised using new razor blades held in parallel 0.5 cm apart.

To test the effects of tissue stiffness on tissue adhesion, some tissue specimens were induced to achieve extreme mechanical states defined as 'compliant' or 'stiff'. The compliant state was induced by incubating the specimens in an artificial seawater solution that was free of Ca^{2+} (Ca^{2+}-chelated ASW). This medium has been shown to be effective for this purpose in previous studies (see Wilkie, 1996). The stiff state was induced by freezing and thawing the tissue specimens, a method shown to be effective in other experiments (G. Szulgit and R. Shadwick, in preparation). Specimens incubated in artificial sea water that included Ca^{2+} (ASW) were considered to be of 'normal' stiffness. ASW consisted of 445 mmol l^{-1} NaCl, 60 mmol l^{-1} $MgCl_2$, 10 mmol l^{-1} KCl, 2.4 mmol l^{-1} $NaHCO_3$, 10 mmol l^{-1} Hepes and 10 mmol l^{-1} $CaCl_2$. The solution of Ca^{2+}-chelated ASW consisted of the same ingredients as ASW, except that 2.5 mmol l^{-1} EGTA (a Ca^{2+} chelator) was added instead of $CaCl_2$. The 2.5 mmol l^{-1} EGTA in these solutions provided an almost 50:1 ratio of chelator to Ca^{2+} to prevent contamination of the solutions by Ca^{2+} endogenous to the tissue specimens. Tissues were never allowed to dry out and were always incubated at 4 °C for 24 h prior to testing, fixing or photographing.

'Freeze-thaw' tissues were placed on a block of dry ice immediately after they had been cut. Once the tissues had been thoroughly frozen, they were thawed in natural sea water at room temperature. Each piece was put through this process three times, taking care to ensure that only one surface of the tissue wafer was in contact with the dry ice and that the opposite surface was used as the testing surface. Transmission electron microscopy confirmed that this procedure disrupted cell membranes, exposing the cell contents to the surrounding tissue (see Results section).

Tissue blocks apposed without pressure

Pieces of dermis were frozen, cut into blocks and placed

side-by-side in a shallow dish containing 20 ml of ASW for 24 h. The tissues were in contact with each other the entire time, but were not pressed together by external forces. These apposed tissues were then pulled away from each other very slightly using forceps. The apposed surfaces tended to remain stuck together while the tissue surrounding these areas was stretched into tendril-like formations, which were photographed through a dissecting microscope. These experiments were conducted in ASW, in Ca^{2+}-chelated ASW and with freeze-thawed tissues.

Microscopy

Freeze-thawed tissues were placed in contact with each other for 24 h without any weight pressing them together. The adhering tissue blocks were then fixed in 2.5 % glutaraldehyde in 0.1 mol l^{-1} sodium phosphate buffer, pH 7.5, gently washed in successive solutions of increasing ethanol concentration (50–100 %) and embedded in Spurr's resin. For optical microscopy, 5 μm thick tissue sections were cut from the interface region between the adhering tissues and stained with Toluidine Blue in 1 % sodium borate. These sections were photographed through a light microscope. For transmission electron microscopy, light gold, ultrathin sections from the same block were stained with lead citrate and uranyl acetate.

Lapshear tests

Specimens for lapshear tests measured 24 mm×10 mm×4 mm, corresponding to the longitudinal, radial and circumferential axes respectively. Tissues were placed in specially designed holders so that they overlapped each other in a controlled fashion and could be handled easily (Fig. 1A). The dimensions of this overlap were measured using calipers to calculate the area of contact. The tissues were then incubated in 150 ml of artificial seawater solution at 4 °C for 24 h (except for those tested after only 2 h). Some of the incubation solutions also included 0.05 % sodium azide as an antiseptic agent. The tissues were stored beneath a 1 g plastic weight to ensure that the surfaces remained in contact for the duration of the incubation (Fig. 1A).

Tissues were taken from their incubation solutions while still in their holders and placed in a T10 tensometer (Fig. 1B). The stabilizing support was then removed from the holder, and the wafers of tissue were separated at a rate of 6 mm s^{-1}. Forces were measured and recorded using the same methods described for determining the adhesive strengths of grafts.

Pieces of cartilage measuring 15 mm×20 mm×2 mm were obtained from a fresh cow patella using the methods described by Reindel *et al.* (1995). These pieces were frozen and thawed using the same methods described for freeze-thawing holothurian dermis (described above) and were then used in lapshear tests.

All statistical analyses were conducted using the software Statgraphics Plus from Manugistics, Inc. (Rockville, MD, USA). Values are reported throughout this paper as means ± standard deviations, unless otherwise indicated. The data represented in Table 1 were pooled from several individuals. Because the data characteristics did not indicate the use of parametric tests, the groups were compared using the Kruskal–Wallis test.

Results

Wounding and grafting

The surface incisions of varying depth appeared to be fully closed within 24–36 h, depending on the depth of the incision, with very little visible scarring. The excision wounds (10 mm wide) were closed within 48 h. The wound closure looked similar to that seen in *Thyone briareus* after 28 days (Menton and Eisen, 1973).

Most grafted tissues stayed in place and appeared to be incorporated into the dermis of the host within 24 h (Fig. 2). Of the twelve pieces of tissue that were autografted, eleven adhered firmly to the wounded surface, judging by the ability of the grafts to remain in place as the animals were handled and by the closure of the host tissue around the outer edges of the graft (Fig. 2). Of the twelve allografts, eight were successful (judged by the same criteria). We suspect that some

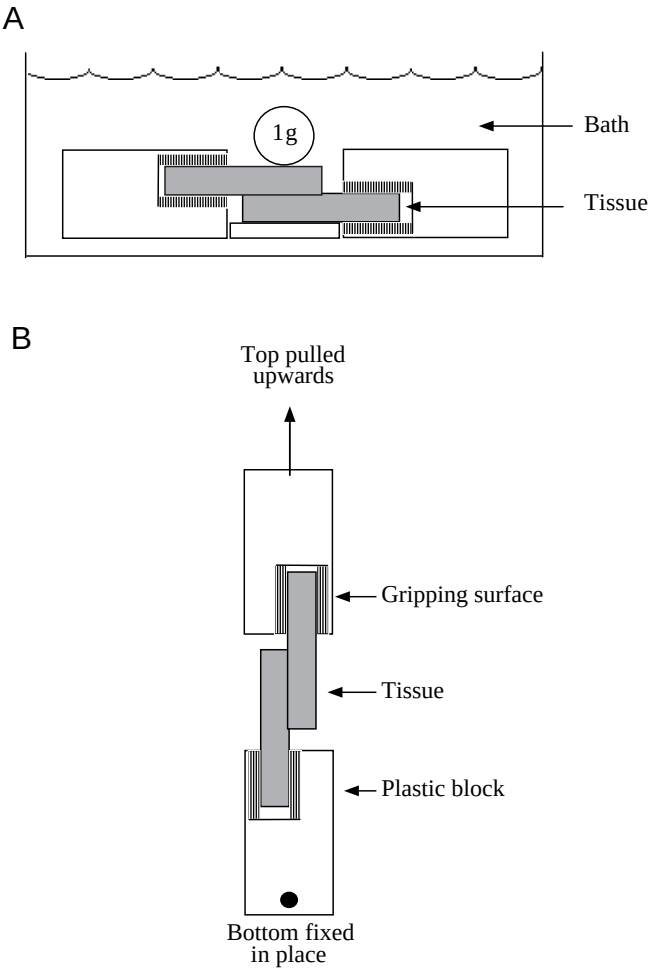

Fig. 1. Schematic diagram of (A) the incubation chamber for the tissues in their holder and (B) tissues about to be drawn apart in a lapshear experiment.

did not ‘take’ because of the natural bending movements of the individuals immediately following their grafting and release. The mean adhesive strength of four of the successful allografts was 13.9±3.1 kPa (equivalent to a shearing force of 200 g on a graft smaller than 1 cm×1.5 cm). The remaining sea cucumbers with allografts were maintained in running sea water to observe the long-term fate of their grafts. Only after 5–6 months did these tissues begin to show obvious evidence of rejection. The original wound appeared to close underneath them in each case, and the grafted tissues were pushed out of their sites over a further period of approximately 2 weeks.

Tissues apposed without pressure

Tissue specimens were placed in contact in a shallow dish of ASW for 24 h without any external pressure pushing them together. In places where adjacent tissues touched, they

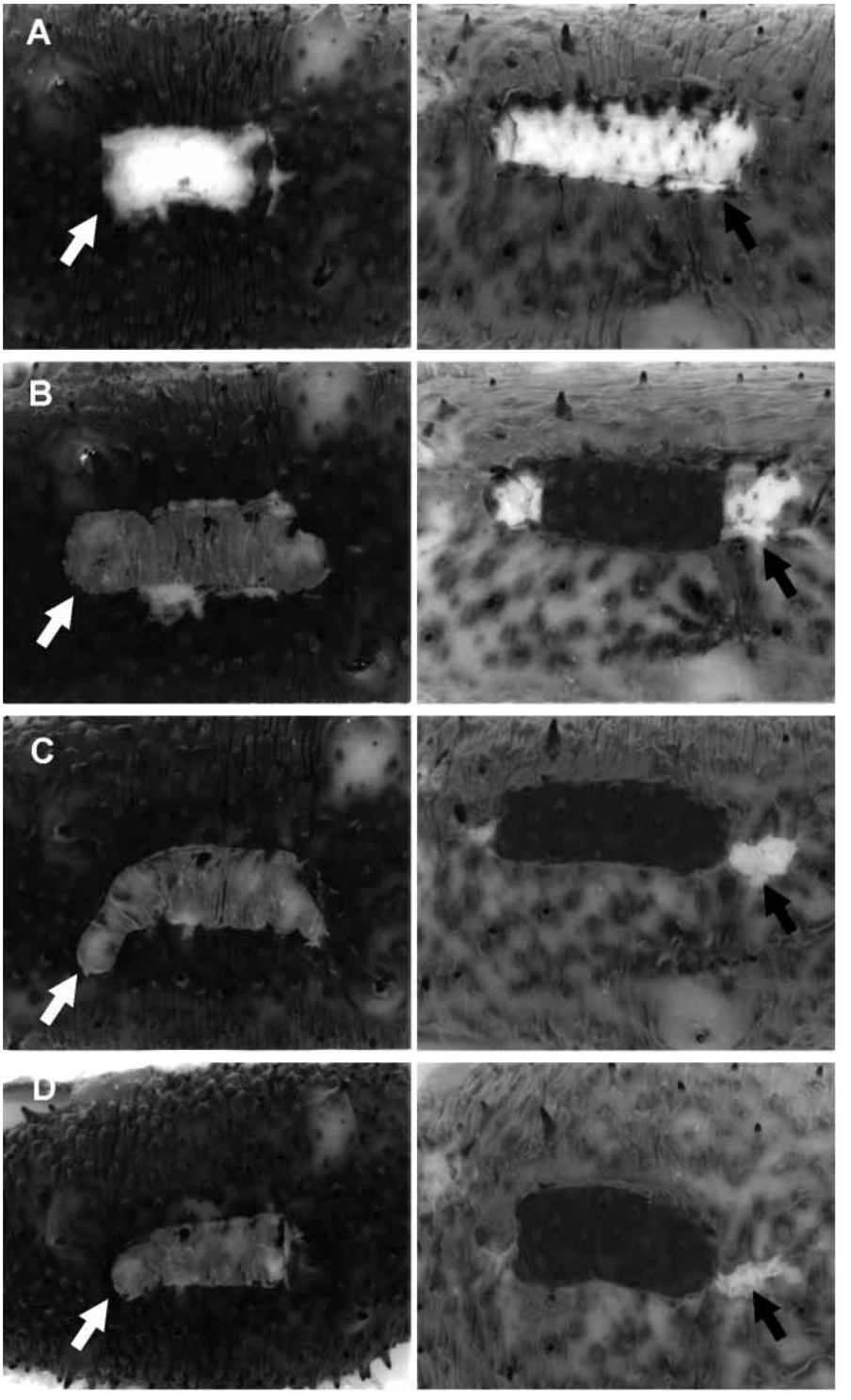

Fig. 2. Allografting of dermal tissue. (A) Intact tissue pieces are excised from two individuals (one shown here on the left and one on the right), leaving wounds indicated by arrows. Note that the wound on the light-colored individual is slightly longer than that on the darker individual. (B) The excised tissue pieces are exchanged. The allografts are not held in place with any bandages, sutures or gels. The white arrow indicates part of an allograft that extends past the wound. The black arrow indicates a wounded area not covered by a graft. (C) 24 h after graft exchange. The epithelial tissue surrounding the graft in each individual has fused with each graft. The grafts have ‘taken’. The graft extension (white arrow) hangs free of the surface and has lost its flat shape. The uncovered area (black arrow) is beginning to close. (D) 48 h after graft exchange. The graft extension (white arrow) has become very soft and can be torn off easily (not shown). The uncovered area (black arrow) has almost fully reapproximated. All pictures are approximately actual size.

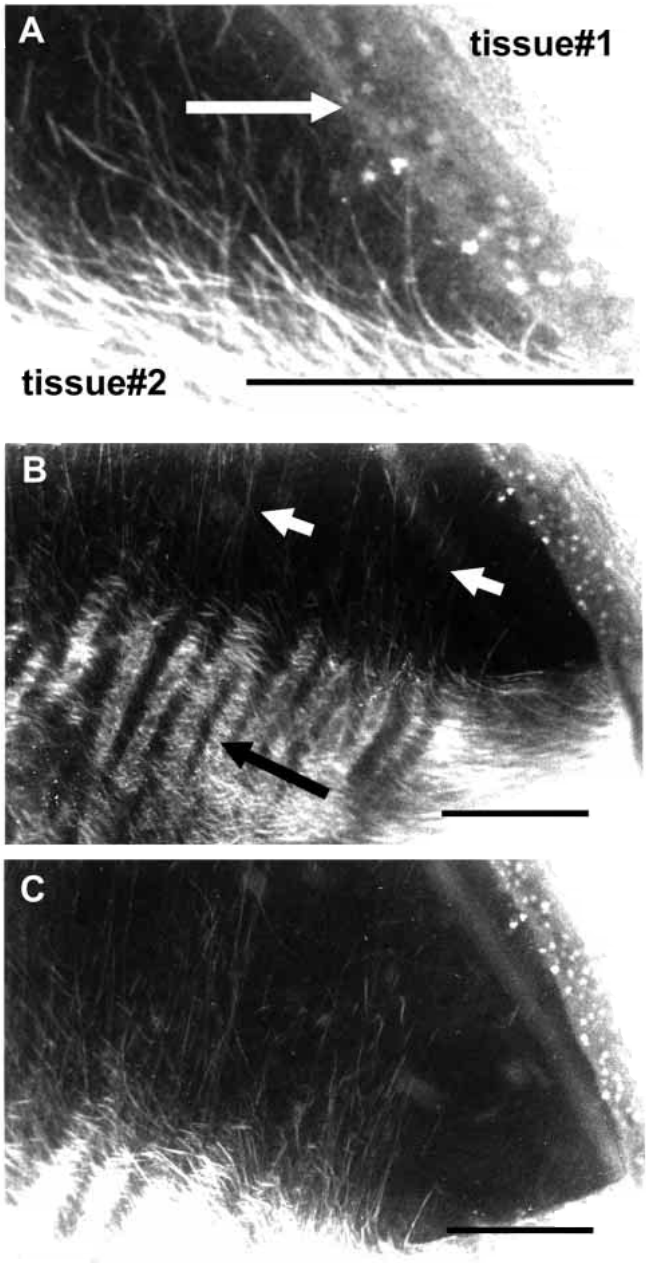

Fig. 3. Apposed, non-pressured tissues are pulled away from each other after adhering. Tissues are being illuminated by both transmitted polarized light and reflected light. (A) Fibers from tissue 1 remain attached to tissue 2 when the two tissues are separated slightly. The white arrow indicates the epidermis of tissue 2, which does not contain fibers. The fibers from tissue 1 are, therefore, adhering to tissue 2 and not simply entangled with fibers from that tissue. (B,C) The same tissues at lower magnification. Forceps (not shown) are used to pull the tissues further apart. As the tissues are separated, the fibers can be seen to be stretched between the tissues (white arrows). Force is transmitted through the fibers, as shown by the stress-induced wrinkles in tissue 1 (black arrow). Scale bars, 1 mm.

adhered. This occurred both in ASW and in Ca^{2+}-chelated ASW, and both in freeze-thawed tissue and in non-freeze-thawed tissue. All portions of the specimens remaining after dissection (epidermis, outer dermis and inner dermis) adhered to all others. The strengths of these adhesions were not measured separately, although the epidermis–epidermis adhesion appeared to be the weakest on the basis of visual inspection while the tissues were separated slightly. Under these conditions, in the inner and outer portions of the dermis, fibrous elements of the tissues persisted in adhering to the apposing surfaces (Fig. 3). These fibrous portions contacted the apposing surfaces with very little surface area, yet they adhered with sufficient tenacity to deform the tissues as they were separated (Fig. 3B). These deformations (wrinkles) persisted as long as the tissues remained separated at a given distance (15 s).

Microscopy

Light microscopy shows that each apposed piece of dermis consists of a collagenous feltwork embedded in a matrix with a sparse cell population (Fig. 4). The collagen fibers did not cross the plane of apposition between the two apposing tissue pieces. The razor-cut ends of the collagen fibers appear distinctly compressed and densely stained. This distinction was used to identify the razor-cut end of a fiber during preparation for transmission electron microscopy (see Fig. 5). The interface between the two tissues is not uniform in appearance. In one region (Fig. 4A,B), the adhesion is so complete that it is difficult to distinguish the interface from the rest of the tissue. These surfaces do not seem to have any distinguishing substance associated with them. In another region of the tissue interface (Fig. 4C,D), a substance with a granular appearance occupies the area between the two wounded surfaces. This substance appears to become more tightly packed as the wounded surfaces draw closer to each other. The regions shown in Fig. 4A,B and Fig. 4C,D, therefore, have different appearances. The reasons for this are not clear.

Fig. 5 is a transmission electron micrograph of the interface between the tissues shown in Fig. 4. The original dissecting cut, which sliced through the collagen, was in an orientation that is now normal to the page (as determined by the distinctively compressed, densely stained appearance of the fiber end). The razor-cut end of the collagen fiber marks the end of one tissue and the beginning of another. The precise location of the interface at this level, however, is not clear. The dominant component of the tissue at this location seems to be an amorphous, granular matrix. There is no visible link between the collagen fiber and any other tissue component; also, no cells gathered at the wound surface.

Lapshear tests

An example of the data collected from a lapshear test is shown in Fig. 6. The first measurements (with the exception of the baseline measurements at time zero) were made after 2 h of tissue-to-tissue contact (Table 1).

Tissues that were stiff adhered just as well as tissues that were compliant. Tissues that had been incubated for 24 h in ASW, Ca^{2+}-chelated ASW or ASW + azide all exhibited similar adhesion responses, as did tissues that had been freeze-

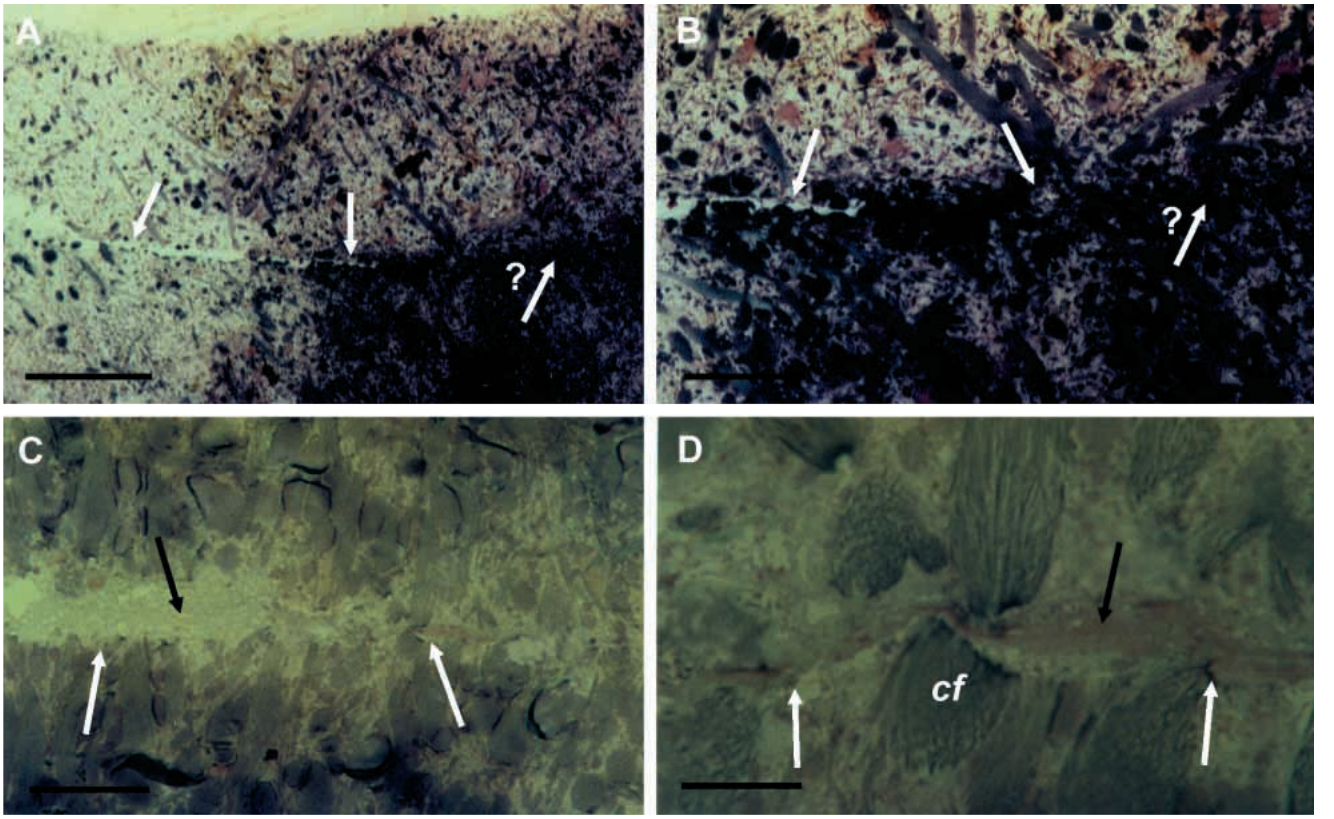

Fig. 4. Light micrographs of the interface between two adhering tissues. (A) The gap between the tissues (white arrows) diminishes until it can no longer be distinguished (arrow with question mark). (B) A higher magnification of A. (C) A different region of the interface between the same tissues. There appears to be a substance with a granular appearance layered between the two tissues (black arrow). The edge of the gap between these tissues is marked by white arrows. (D) C at higher magnification. Note that the collagen fibers (*cf*) do not cross the gap between tissues to entangle with other fibers. Scale bars, A, 250 μm; B, 100 μm; C, 50 μm; D, 10 μm.

thawed (Table 1). The tissues incubated in Ca^{2+}-chelated ASW were noticeably less stiff than those that had been incubated in ASW. The tissues that had been freeze-thawed were very stiff.

Dermal tissues placed against glass slides or cow patellar cartilage adhered relatively weakly (Table 1). Both these surfaces were smooth and stiff, although their chemical compositions were obviously quite different. We were unable to measure any adhesion between apposed pieces of cartilage.

Discussion

Wound reapproximation

Complex organisms have developed various ways of mending themselves when they suffer injuries. The first step usually involves stabilizing the damage, which is then followed by a reconstruction of the wounded site at the cellular level. This reconstruction is often slow and can be subject to problems such as infection or reinstatement of the wound through mechanical stresses. Because reconstruction depends largely on the volume of tissue that needs to be replaced, reapproximating the wound (i.e. bringing the displaced surfaces back into their original alignment to reduce the wound space) can greatly aid the healing process. Humans usually use some sort of tool to accomplish this (e.g. sutures, tapes, staples, etc.). Once reapproximated, most vertebrate tissues can heal rather quickly if they are well vascularized. Highly collagenous tissue such as that found in the cartilage or ligaments of vertebrates, however, is very poorly vascularized and does not heal quickly. This was demonstrated using blocks of cow patellar cartilage (Reindel *et al.* 1995).

The dermis of *P. parvimensis* is interesting because it is able to accomplish reapproximation so easily, apparently because of its composition. Furthermore, although it resembles human tissues that are highly collagenous and minimally vascularized, it heals extremely well and is able to maintain live grafts for extended periods. While holothurian dermis is not exactly homologous to vertebrate dermis (Holland, 1984), it consists largely of proteins that are extremely similar to those in our own connective tissues and it contains a sparse assortment of cells (including nerves). A major component is collagen, which exists in the form of bipolar, spindle-shaped fibrils 39–436 μm long (Trotter *et al.* 1994) that have proteoglycans associated with their surfaces (Scott, 1988, 1991; Trotter and Koob, 1989; Trotter *et al.* 1995). The fibrils are further organized into larger structures known as fibers. These fibers are loosely arranged, appearing as a feltwork pattern under the microscope (Figs 3,

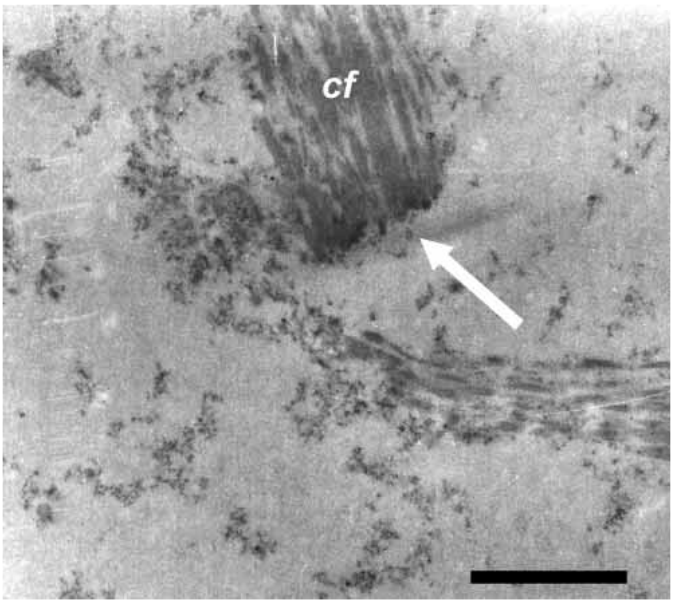

Fig. 5. A transmission electron micrograph of the interface between two adhering surfaces. The arrow indicates the end of a collagen fiber (*cf*), which marks the original razor cut through the tissue and should determine its boundary (the interface between the two tissues). There is no clear boundary, however, only an apparent continuum of extracellular matrix. Scale bar, 2 μm.

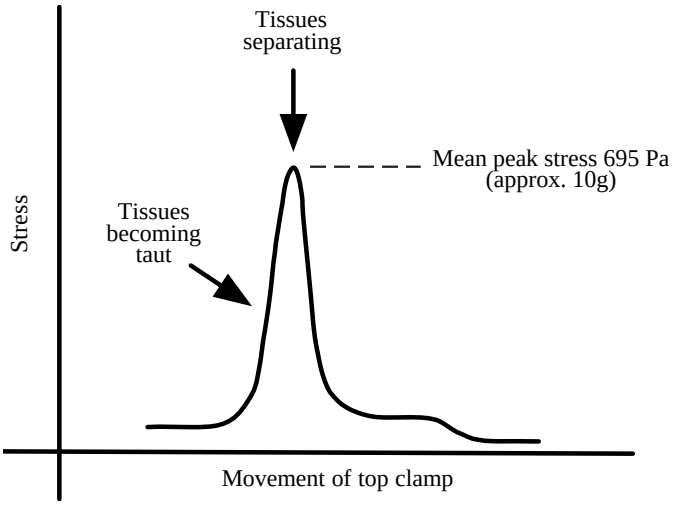

Fig. 6. Representative data plotted as stress *versus* movement of the tissue holder. The peak stress represents the strength of the join between the tissues. The abscissa does not depict strain (the usual convention) because the actual join is not being strained appreciably in these tests (it is either intact or not). Instead, tissue above and below the join is allowed to stretch while the join remains intact.

4). There is also an extensive, non-collagenous microfibrillar network that permeates the dermis (Thurmond and Trotter, 1996) and may be very important in its mechanical behavior. Finally, all these components reside in an apparently amorphous extracellular matrix (Figs 4, 5).

The healing ability of holothurian dermis has not been extensively investigated. There are a few studies that have focused on the cellular response to tissue insult, but they do not discuss wound closure prior to this response (Cowden, 1968; Menton and Eisen, 1973). An exception to this is the work of Fankboner (1978), who describes 'self-suturing' in *Opheodesoma spectabilis* by means of calcified ossicles in the dermis. We could find no such structures in the dermis of *P. parvimensis*. Cowden (1968) made incisions in the dermis of *Stichopus badionotus* and noted that the tissue became reapproximated after 6–8 h, but gave no details of this initial stage of closure. The rest of his observations describe the healing process at least 2 days after the incision had been made. Menton and Eisen (1973) describe the response of *Thyone briareus* dermis to excision. They note that 1 cm wide excised wounds take 28 days to reapproximate. The huge discrepancy between this result and the reapproximation time measured in *P. parvimensis* is not understood at this point, but we speculate that the excised dermis of *P. parvimensis* is more easily pulled together initially by the subdermal muscles.

Studies of the clotting ability of holothurian coelomic fluid (see Smith, 1981, for an excellent review of work prior to 1981; see also Canicatti *et al.* 1989, 1992; Canicatti and Farina-Lapari, 1990) have focused mostly on the structure, classification and clotting behavior of various coelomocytes. We have no doubt that coelomocytes are important to the long-term healing process in holothurians and may contribute to the healing that we see *in vivo*. Our *in vitro* freeze-thaw experiments, however, demonstrate that the initial adhesion of isolated pieces of dermis does not require viable cells. The reason(s) for the huge difference in shear breaking stresses between *in vivo* and lapshear adhesions (Table 1) is not known, but may include the activities of coelomocytes and/or other cells.

Grafting has previously been accomplished in holothurians (specifically *Cucumaria tricolor*) by Hildemann and Dix (1972). In their study, sutures were required to hold the grafts in place while they healed, after an absorbable gelatin had been dusted onto the graft sites to form temporary gelatinous seals. We suspect that the grafts in our study did not require sutures or gelatin because of the malleable nature of the dermis and because of the adhesive nature of the viscous fluid on the wounded surface. Hildemann and Dix (1972) report that *C. tricolor* has a 'leathery skin'. This 'leathery skin' may not creep as well as the soft dermis of *P. parvimensis*. The sealing that was required for *C. tricolor* grafts was not necessary with *P. parvimensis*, probably because the 'floppy' nature of the grafted tissues allowed them to fit well against the wounded surface (although not to the microscopic level described below). The eventual graft rejection that was seen in our study was also seen and very well documented by Hildemann and Dix (1972), although the tissues in their study were rejected sooner (approximately 3–4 months after grafting).

Does the mutability of the dermis affect adhesion?

One unique feature of echinoderms is that they can rapidly and reversibly alter the stiffness of their connective tissues by

Table 1. *Adhesion between dermal tissue specimens in various incubation solutions reported as mean breaking stresses*

Incubation solution	Breaking stress of join between specimens (Pa)	*N*	Mechanical state of specimens
In vivo grafts	13900±3600	4	Normal
ASW	695±321	31	Normal
ASW, freeze-thawed	393±211	9	Stiff
Ca^{2+}-chelated	505±215	7	Floppy
Ca^{2+}-chelated, freeze-thawed	487±164	6	Stiff
ASW, 2 h	205±59	5	Normal
ASW freeze-thawed, 2 h	277±101	10	Stiff
Ca^{2+}-chelated, 2 h	495±286	9	Floppy
ASW + azide	613±362	20	Normal
ASW on cartilage	129±100	4	Normal
ASW on glass	124±28	4	Normal

Values are means ± S.D.

ASW, artificial sea water.

All incubations lasted 24 h unless otherwise stated.

The mechanical state of tissue specimens was determined by visual observation during handling.

Note that stiffness does not correlate with breaking stress.

Only values for tissues placed against cartilage and glass differed significantly from the ASW controls (P<0.05).

orders of magnitude (see Wilkie, 1996, for an excellent review). The tissues can have a stiffness similar to that of mammalian connective tissues one moment, only to become extremely soft and compliant a few moments later. The phenomenon has been shown to be neurally mediated (Maeda, 1978) and requires the presence of intact cells (Szulgit and Shadwick, 1994; Trotter and Koob, 1994, 1995). Tissues that have had their cells perforated with the detergent Triton X-100 become irreversibly stiff, even in the presence of a Ca^{2+} chelator (Szulgit and Shadwick, 1994). This has led some researchers to believe that there is a matrix-stiffening agent within certain yet-to-be identified dermal cells that is released in cases of cell perforation or lysis and is prevented from being released when Ca^{2+} is not present (Szulgit and Shadwick, 1994; Trotter and Koob, 1995). Our current observation that freeze-thawed tissues become stiff, even in the presence of a Ca^{2+} chelator, is consistent with this idea.

Because collagen fibrils inside the tissue do not stretch appreciably when the tissue is lengthened (Smith, 1981; Hidaka and Takahashi, 1983), it is generally thought that the fibrils slide past each other under these conditions (conversely, the fibrils are thought not to slide past each other when the tissue is stiff). This fibrillar sliding allows the tissue to deform plastically over time if subjected to a mechanical stress; a property known as ‘creep’. This property was apparent when 1 cm×1.5 cm pieces of tissue were placed on rough surfaces for several hours beneath 1 g weights. The bottom surface of the tissues deformed (crept) to match the intricacies of the surfaces below (G. K. Szulgit and R. E. Shadwick, personal observation). Waite (1983) and Denny (1988) provide succinct descriptions of some of the factors that promote adhesion and note that close physical contact is one of the most important factors in maximizing the potential of an intervening adhesive (for a more thorough discussion of adhesion, see Israelachvili, 1991). Because sea cucumber dermis is known to creep, the two wounded surfaces can theoretically flow together to match each other almost perfectly, on a microscopic scale, with very little space between them. We measured the distance between surfaces to be 3.6 μm in one area (Fig. 4C,D) and to be immeasurably small (using our techniques) in another area (Fig. 4A,B). This proximity should allow for maximum wound closing potential of an adhesive substance between the surfaces. In this sense, we would expect the mutability of the dermis to be extremely important in the initial reapproximation of the wounded surfaces because it should cause a quicker onset of adhesion in tissues that can creep more rapidly (i.e. when the tissue is compliant). When we compared the adhesion of stiff and compliant tissues, however, we found no significant difference in adhesion between the two mechanical states after 2 h of contact or 24 h of contact (Table 1).

What are the possible methods of adhesion?

There are many factors that could be involved in the adhesion between tissue pieces. For the purpose of this paper, these will be divided into two over-simplified categories: ‘physical’ and ‘chemical’. The physical forms of adhesion include mechanical linkages, capillary adhesion and viscous shear. The chemical forms refer to any attraction that is caused by electrostatic interactions (e.g. Van der Waals interactions, ionic bonding, hydrogen bonding). The contributions from each of these factors are considered below.

Mechanical adhesion

The contribution due to interlinking surfaces appears to be minimal. Collagenous fibers at the surface of the wound did not become entangled with fibers from the apposing surface (Fig. 4), although there may be entanglements between structures that are not visible at this level of magnification.

Capillary adhesion

The force required to create a new air–water interface as the wetted tissues were separated was measured by placing wetted tissues together for 5 s and then pulling them apart. This allowed the water from apposing surfaces to cohere, but did not allow the tissue surfaces to creep close together on a micro scale. The forces in these tests were immeasurably small using our techniques. Furthermore, the forces reported from the lapshear tests were peak forces, recorded just prior to the tissues actually separating (Fig. 6). At the time that the forces were recorded, therefore, there was no new air–water interface being created.

Viscous shear stress

Shearing a fluid between two parallel surfaces requires a stress described by the equation:

$$\tau = \mu U/z,$$

where τ is shear stress, μ is the dynamic viscosity of the fluid, U is shear velocity and z is the distance between the surfaces.

Excised pieces of dermis quickly develop some kind of viscous fluid on their surfaces. Fig. 4C,D shows a substance with a granular appearance in the gap between wounded tissue surfaces, and this viscous fluid might be responsible for adhesion between tissue pieces. If so, this raises the question of whether the adhesion is physical or chemical. If the viscous fluid has no particular chemical attraction to the tissues, then pulling the tissues apart (thus shearing the fluid between them) could still require a significant force (where the intermolecular forces and the extent of entanglements between molecular chains produced very high values of μ). If this were the case, however, one would expect similar levels of adhesion between the tissues and any other surface. This is clearly not the case, because cartilage and glass adhere to the tissues with very little tenacity. Viscous shear forces also fail to explain the prolonged adhesion under stress between apposed tissues (Fig. 3B). The contact areas between the surfaces (fibers) in these tissues are extremely small, which means that the stresses on them will be quite large for a given separating force. If viscous adhesion were the only force sticking them together, they should become unstuck after the application of a stress (even a small one) for an extended period (15 s), but this did not occur.

Chemical adhesion

Adhesion between tissue specimens that have been in apposed, non-pressured contact is probably aided only minimally by the mutability of the tissue, because there is very little force to cause the tissue to creep. In these cases, it is evident that only a very small area of contact between the tissues is necessary to achieve adhesion and that the adhesion is probably based on something other than viscous shear between two parallel surfaces. That they do not become unstuck suggests some form of adhesion based on weak chemical bonds.

Are there any other known adhesives in echinoderms?

Adhesive secretory substances, common in the podia and cuvierian tubules of holothurians, are thought to be mixtures of proteins and mucopolysaccharides that are secreted from specialized cells and adhere to a wide variety of surfaces (Flammang, 1996). The nature and origin of the adhesive in our study, however, remain unclear. In tests where the tissues were frozen and thawed, the cells in the tissues were presumably lysed, precluding them from participating in any kind of metabolically active healing response. Substances previously contained within these cells, however, could have diffused to the wound surface. In other tests, the cellular activity in unfrozen tissues was presumably halted by the addition of 0.1 % azide (a metabolic blocker). This prevented the cells from actively participating in the healing process while also presumably reducing their secretory ability. We did not test the secretory ability of poisoned cells, but we did determine that the tissue specimens were much less stiff that those that had been freeze-thawed. This indicates that there was a definite difference between the two treatments. We attribute this difference to a reduction of active cellular secretions. If one assumes that most of the cells in these tissues were intact after dissection, then their contents would have remained locked within them and would have had no effect on the extracellular matrix. Undoubtedly, a few cells were ruptured at the wound surface where the original cuts were made (microscopy reveals that approximately 1 % of the cross-sectional area of the tissue, at this depth, is composed of cells). On the basis of this evidence, it seems more likely that the adhesive substance is not secreted near the wound site at all, but is part of the amorphous matrix substance that occurs at all times between fibers in the dermis (Figs 4, 5).

Under what conditions is the adhesive effective?

Is the adhesive in our study simply a highly charged substance that sticks to anything, having no special affinity for holothurian tissue? Or does it preferentially adhere to some component of the dermis? The tissue sticks only weakly to glass and cartilage, yet it sticks to itself quite well, demonstrating that it has some level of specificity. There are several known adhesive molecules (e.g. fibronectin, laminin and cadherins) that hold vertebrate tissue components together in normal, healthy tissue. If holothurian tissues also possess these proteins, or similar proteins, then these might link with other components of each surface (and/or themselves) in wounded tissue. Trotter *et al.* (1996) demonstrated that collagen fibrils can be isolated from the dermis of the sea cucumber *Cucumaria frondosa* simply by washing out some of the dermal components. One of these components can then be added back to the isolated fibrils causing them to flocculate. They have named this component ‘stiparin’. What stiparin is doing to the fibrils, to the surface proteoglycans or to the ionic environment surrounding them remains unclear. Perhaps further elucidation of the role of stiparin in isolated fibril flocculations of *C. frondosa* will provide some information regarding adhesion between wounded dermal surfaces in *P. parvimensis*.

When speculating on the specificity of the chemical bonds involved in this adhesion, it is important to remember that adhesion occurs in the absence of Ca^{2+} (Table 1). This treatment has dramatic mechanical effects on the dermis, indicating that it affects the interactions between the components in some way. The details of these interactions are still undetermined, but it is interesting that the mechanical effects induced by the removal of Ca^{2+} appear not to be linked to any changes in the adhesive properties of the tissue.

It is our hope that future work with holothurians will explore applications of this tissue for tissue grafting technology. Artificial skins are becoming increasingly popular as temporary and permanent wound dressings for traumas such as burns. These often consist of synthetic meshworks (Aliabadi-Wahle *et al.* 1996) in which fibroblasts are sometimes cultured (Hansbrough *et al.* 1994). Much of the

research on tissue grafting is concerned with getting the graft to ‘take’ (to adhere to the wounded area long enough to promote healing). This does not seem to be problematic for *P. parvimensis* dermal grafts. One of the most interesting aspects of the allografts is that they survive even though they are 3 mm thick or thicker (this is much thicker than typical human skin grafts), as long as they are directly above the wounded area. If part of the graft hangs over the edge of the wound (Fig. 2), then that portion of the graft rapidly atrophies. This implies that the grafted tissue is being kept alive by something other than the surrounding sea water (i.e. the host holothurian). Do substances migrate from the host to the graft tissue and, if so, why do they stop at the overhanging region? Is it due to a simple concentration gradient of migrating substances along the graft, to bacterial attack of sparsely vascularized regions of the graft or to something more complicated? The exceptional grafting ability and non-cellular adhesion of *P. parvimensis* dermis might lead to important insights in other areas of grafting research.

In conclusion, a surprising result of the present study is the uniformity of the adhesion response of *P. parvimensis* dermis, irrespective of its incubation conditions. Tissues adhered to each other whether cells were intact, had been lysed by freezing or had been treated with a metabolic blocker. The adhesion, therefore, is not a product of cellular activity, but appears to be due to the composition of the extracellular matrix. The adhesion is more effective between dermal specimens than it is between dermis and glass or vertebrate cartilage and seems to be a product of chemical forces as opposed to physical forces. The mechanical state of the tissue, as discussed in many other papers (Wilkie, 1996), does not seem to affect the magnitude of tissue adhesion. Frozen and thawed tissues are orders of magnitude stiffer than those incubated in Ca^{2+}-chelated ASW (G. K. Szulgit and R. E. Shadwick, in preparation) yet they all have similar adhesion capabilities.

For holothurians, and echinoderms in general, there are aspects of connective tissue composition or design that have not yet been seen in other phyla, either because they do not exist or because they are well hidden in other animals. If the latter is true, sea cucumbers should become increasingly important as a model system in the field of extracellular matrix biology.

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References

ALIABADI-WAHLE, S., CHOE, E. U., JACOB-LABARRE, J., FLINT, L. M. AND FERRARA, J. J. (1996). Evaluation of a novel synthetic material for closure of large abdominal wall defects. *Surgery* **119**, 141–145.

CANICATTI, C. AND FARINA-LAPARI, E. (1990). Dynamic and morphological aspects of coelomocyte clotting in *Holothuria polii*. *J. Invertebrate Path.* **56**, 63–69.

CANICATTI, C., MIGLIETTA, A. AND COOPER, E. L. (1989). *In vitro* release of biological active molecules during the clotting reaction in *Holothuria polii*. *Comp. Biochem. Physiol.* **94**A, 483–488.

CANICATTI, C., RIZZO, A. AND MONTINARI, M. R. (1992). Adhesive properties of sea cucumber coelomocytes. *Biol. Cell* **76**, 97–102.

COWDEN, R. R. (1968). Cytological and histochemical observations on connective tissue cells and cutaneous wound healing in the sea cucumber *Stichopus badinotus*. *J. Invertebrate Path.* **10**, 151–159.

DENNY, M. W. (1988). *Biology and the Mechanics of the Wave-Swept Environment*. Princeton, NJ: Princeton University Press. 304pp.

FANKBONER, P. V. (1978). Self-suturing by a synaptid sea cucumber (Holothuroidea: Echinodermata). *Experientia* **34**, 729–730.

FLAMMANG, P. (1996). Adhesion in echinoderms. *Echinoderm Studies* **5**, 1–60.

HANSBROUGH, J. F., MORGAN, J., GREENLEAF, G. AND UNDERWOOD, J. (1994). Development of a temporary living skin replacement composed of human neonatal fibroblasts cultured in Biobrane, a synthetic dressing material. *Surgery* **115**, 633–644.

HIDAKA, M. AND TAKAHASHI, K. (1983). Fine structure and mechanical properties of the catch apparatus of the sea-urchin spine, a collagenous connective tissue with muscle-like holding capacity. *J. exp. Biol.* **103**, 1–14.

HILDEMANN, W. H., BIGGER, C. H. AND JOHNSTON, I. S. (1979). Histocompatibility reactions and allogeneic polymorphism among invertebrates. *Transplant. Proc.* **11**, 1136–1142.

HILDEMANN, W. H. AND DIX, T. G. (1972). Transplantation reactions of tropical Australian Echinoderms. *Transplantation* **15**, 624–633.

HOLLAND, N. (1984). Epidermal cells. In *Biology of the Integumen*t, vol. 1, *Invertebrate*s, chapter 14 (ed. J. Bereiter-Hann, A. G. Matoltsy and K. S. Richards), pp. 756–789. Berlin: Springer-Verlag.

HYMAN, L. H. (1955). *The Invertebrates.* IV. *Echinodermata.* New York: McGraw-Hill.

ISRAELACHVILI, J. N. (1991). *Intermolecular and Surface Force*. San Diego: Academic Press. 450pp.

MAEDA, R. (1978). Nervous control of coordination in the catch apparatus in sea-urchin spines. MSc thesis, Tokyo: University of Tokyo. 60pp.

MENTON, D. N. AND EISEN, A. Z. (1973). Cutaneous wound healing in the sea cucumber, *Thyone briareus*. *J. Morph.* **141**, 185–204.

REINDEL, E. S., AYROSO, A. M., CHEN, A. C., CHUN, D. M., SCHINAGL, R. M. AND SAH, R. L. (1995). Integrative repair of articular cartilage *in vitro:* Adhesive strength of the interface region. *J. orthoped. Res.* **13**, 751–760.

SCOTT, J. E. (1988). Proteoglycan–fibrillar collagen interactions. *Biochem. J.* **252**, 313–323.

SCOTT, J. E. (1991). Proteoglycan:collagen interactions in connective tissues. Ultrastructural, biochemical, functional and evolutionary aspects. *Int. J. Biol. Macromol.* **13**, 157–161.

SMITH, V. J. (1981). The echinoderms. In *Invertebrate Blood Cells*, vol. 2 (ed. N. A. Ratcliffe and A. F. Rowley), pp. 513–562. London: Academic Press.

SZULGIT, G. K. AND SHADWICK, R. E. (1994). The effects of calcium chelation and cell perforation on the mechanical properties of sea urchin ligaments. In *Echinoderms Through Time: Proceedings of the Eighth International Echinoderm Conference* (ed. B. David), pp. 887–892. Rotterdam: A. A. Balkema.

THURMOND, F. A. AND TROTTER, J. A. (1996). Morphology and biomechanics of the microfibrillar network of sea cucumber dermis. *J. exp. Biol.* **199**, 1817–1828.

TORELLE, E. (1909). Regeneration in Holothuria. *Zool. Anz.* **35**, 15–22.

TROTTER, J. A. AND KOOB, T. J. (1989). Collagen and proteoglycan in a sea urchin ligament with mutable mechanical properties. *Cell Tissue Res.* **258**, 527–539.

TROTTER, J. A. AND KOOB, T. J. (1994). Biochemical characterization of fibrillar collagen from the mutable spine ligament of the sea-urchin *Eucidaris tribuloides*. *Comp. Biochem. Physiol.* **107**B, 125–134.

TROTTER, J. A. AND KOOB, T. J. (1995). Evidence that calcium-dependent cellular processes are involved in the stiffening response of holothurian dermis and that dermal cells contain an organic stiffening factor. *J. exp. Biol.* **198**, 1951–1961.

TROTTER, J. A., LYONS-LEVY, G., LUNA, D., KOOB, T. J., KEENE, D. R. AND ATKINSON, M. A. L. (1996). Stiparin: A glycoprotein from sea cucumber dermis that aggregates collagen fibrils. *Matrix Biol.* **15**, 99–110.

TROTTER, J. A., LYONS-LEVY, G., THURMOND, F. A. AND KOOB, T. J. (1995). Covalent composition of collagen fibrils from the dermis of the sea cucumber, *Cucumaria frondosa*, a tissue with mutable mechanical properties. *Comp. Biochem. Physiol.* **112**A, 463–478.

TROTTER, J. A., THURMOND, F. A. AND KOOB, T. J. (1994). Molecular structure and functional morphology of echinoderm collagen fibrils. *Cell Tissue Res.* **258**, 451–458.

WAITE, J. H. (1983). Adhesion in byssally attached bivalves. *Biol. Rev.* **58**, 209–231.

WILKIE, I. C. (1996). Mutable collagenous tissues: Extracellular matrix as mechano-effector. *Echinoderm Studies* **5**, 61–102.