From Rob KASS To Dr G. MAJNO - June 21, 1972

NOTES ON THE TURTLE EXPERIMENTS

The purpose of the turtle experiments was to provide an in vitro explanation of the observed phenomenon that the healing of a wound is initiated in its corners. The problem became one of creating such a wound in cultured monolayers of fibroblasts, and creating it in an observable and relevant situation. The chosen technique was very successful, except that the most aesthetic qualities have yet to be realized. Briefly, small pieces of Parafilm were flattened on the bottoms of petri dishes (or on Sykes-Moore coverslips in petri dishes). These were cut into various polygonal shapes, usually squares, with emphasis on the quality of the corners. The dishes were then sterilized and fibroblast cultures set-up. After anywhere from 18 to 60 hours, the squares were carefully removed with the result that clean geometric "wounds" were left in the monolayers.

METHOD

There are several points worth mentioning in describing this technique.

First, the choice of fibroblasts for culture. It became evident that rat sub-dermal tissue, admittedly an ideal choice, was inferior in culture. Perhaps embryonic tissue would be better, but apparently rat tissue of any origin is difficult to work with in culture. An easy, reliable choice is mouse embryo tissue, with fibroblasts being extracted in the following way:

animal killed by exsanguination (decapitation)

uterus aseptically removed

embryos aseptically removed from uterus

sterility:

- embryos cut into small pieces in beaker with scissors (ten min.; approx. 1m
- Pieces rinsed three times in medium (or saline) to remove blood
- pieces trypsinized for 15 min, the trypsin poured off through three layers of gauze and stored in refrigerator (this was done 3 times)
- cells centrifuged from trypsin then resuspended in medium (C-135, 10% new-born calf serum) and put in 4 "GE 500" bottles at 37°C.
- after one (or two) day the bottles were shaken to remove non-fibroblasts and the fluid changed.

The second consideration is the cutting of the squares in the parafilm in the petri dishes. The objective is to produce neat clean corners in the parafilm while being sure that the corner which is cut remains firmly adhered to the plastic or glass which will become the substrate. Further, it is useful, and in fact necessary, to know the exact original position of the square (except when using continuous viewing as with the time-lapse photographic experiments). This is why the parafilm was cut into squares after being adhered to the dishes rather than before [the first experiment was inadequate just for this reason]. In this way a cut inthe plastic was also made and in the same place as the edge of the parafilm so that growth could be observed at will with reference to the original wound always present. This method introduced an additional artifact, but one which is not objectionable and may even be desirable: the cuts, which were in reality ditches, made in the plastic present a substantial barrier to the ingrowing fibroblasts in that they must extend at least part of their cytoplasm down and then up the "ditch" in order to get across*. In imagining the situation in vivo where the cells immigrating into the wound may have

* see "addendum" at end of text!

a similar hurdle (in climbing down into the wound), the ditches may even help to approximate this situation.

Along with the advantage of having the "ditches" to mark the edges of the square, goes the problem of the aesthetic qualities of the system. The tool used to cut the edges was a piece of razor blade fastened by a washer and screw to a small plexiglass dowel. In order to assure a firm cut of the parafilm so it would stay adhered to the surface, the cutting strokes had to overlap some. On glass this made no difference but on plastic this meant that the ditches also would overlap. This did not have any effect on the growth pattern of the cells, but it had an untidy appearance. So at least for photographic purposes a solution should be found. The obvious choice is to find a cutting instrument which would assure a perfect corner in both parafilm and plastic.

The size of the squares varied from 1/10 to 5 square millimeters. The best size was around 1 square mm. However, when it is only thecorners in question, the remainder of the polygon is irrelevant; all that is important is that the corner is a true corner, and enough parafilm is spread to prevent cells from growing in from the open side rather than the edges.

As far as adhesion of the parafilm to the plastic or glass is concerned, the pieces were firmly pressed onto the surface with a rounded edge of a plexiglass or wooden dowel. When there was any doubt as to their adhesion they were tested by rinsing with alcohol before the cutting of the squares took place.

One last point about the cutting process is that it must be remembered that the plastic is very soft indeed and the slightest scratch will often show up as an ugly gash at the microscopic level. This is especially important when lifting away excess parafilm around the cut square. Similarly, the cutting of the edges must be done gently to avoid disfiguration of

the plastic around the square.

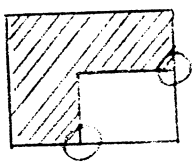
Next, after the petri dishes and/or coverslips are ready they must be sterilized. This was done with alcohol which was allowed to dry completely beneath an ultra-violet light source. The petri dishes were then seeded to provide confluent monolayers within 48 hours (sometimes sooner). When the monolayers were fully confluent, the squares of parafilm were sterily, and carefully lifted (they had to be lifted vertically to avoid damaging the surrounding cells).

Finally, when photographs were to be taken, it was found best to clean the bottom of the petri dish, change the medium to rinse debris away, and to remove the cover of the dish to avoid blurring of the image. The 16x objective was optimal on the inverted Zeiss phase-contrast microscope.

CONTROLS

There were two types of controls which were tried. The first was to replace the fibroblasts by HEp-2 cells. The results were absolutely striking. They exhibited none of the re-orientation and growth in the corners of the squares which was characteristic of the fibroblasts.

The second was not particularly successful. It consisted of parafilm being left around two sides of the square so that in two corners cells grew in in only one direction.



This was to control the contact phenomenon. The results of those done indicated that they should be repeated, but as things stood, they were not conclusive. The lack of success was due to not enough shapes of high enough quality being cut.

THREE POINTS

A slight variation in the technique is provided by changing the angle of the corners, both acute and obtuse. These were each tried and will surely present pretty pictures, perhaps adding some strength to observations using right angles.

Also circular wounds, after early difficulties, were never returned to. It might well be worth again trying to cut circles, for the purpose of comparison.

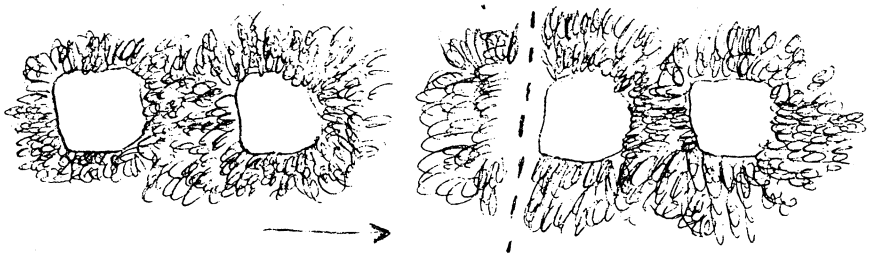
One other observation which could at some time be useful, was that the fibroblasts appear to grow right over the parafilm. This could be useful if an artificial control or some such thing was desirable for explant experiments since cells did grow (once) from an "explanted" piece of parafilm when the piece was removed from the monolayer and placed in a fresh petri dish with medium.

TENSION EXPERIMENTS

There is another, related experiment involving fibroblasts in culture. This uses the well-known phenomenon that there is substantial tension in sheets of fibroblasts in culture. If explants are placed near each other, the tension in the outgrowing fibroblasts is sufficient to pull the pieces of tissue together. Ruth first described this in 1911 and James and Taylor used this observation to measure the tension developed by the fibroblasts in such a system. They used chick frontal bone explants, but the origin is unimportant since in repeating the experiment myself I used mouse embryo heart explants which were available on account of another experiment. If explants of approximately 1 square mm are placed about one mm apart and the

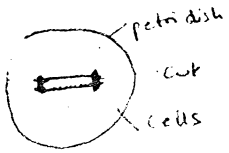
confluent outgrowth of fibroblasts is physically cut from the three non-opposing sides of one of the pieces of tissue, the piece is rather quickly drawn to the other.

As it stands the method probably would have little use in testing pharmacological agents. Since contraction induced by serotonin is "of the order of three percent" a sensitive system with an accurate method of measuring is needed. If from only one side of one piece of tissue, was separated the outgrowth of fibroblasts, then certainly some approaching of one piece to the other would occur:



It would be insufficient to bring the two pieces together, however, and so an accurate measurement of movement could be made and drugs such as serotonin could be added.

A similar method would utilize only the confluent sheet of cells themselves. If a long, thin strip was separated from the rest of the sheet of fibroblasts in a petri dish it would retract away a little, but then there would remain a miniature strip of tissue, much like a strip of granulation tissue in the microchamber, which could easily be measured for action under pharmacological agents. I think a strip nearly the diameter of the petri dish and as narrow as possible would provide the most sensible way of testing such agents, since in that way the contracting action is mostly along one axis as it is in the tests of granulation tissue.



A piece of a razor blade would be a suitable instrument to make such a cut. As far as measuring contraction is concerned,

in either case I imagine a calibrated eye piece would be the easiest. Otherwise shallow marks could easily and accurately be cut in the bottom of the petri dish at half-millimeter intervals before seeding with cells. A three percent contraction would mean a little over one such unit.

At any rate there is no doubt in my mind that the latter system, a strip cut in a monolayer [which could be made neater, if desired, by using a mold of parafilm] would show drug-stimulated contraction, provided that cells of the right age and degree of confluency were used.

STATEMENT

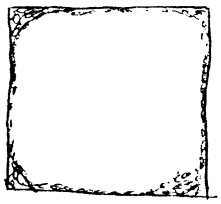
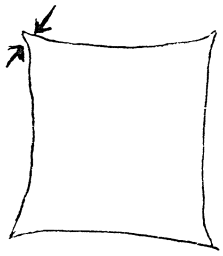
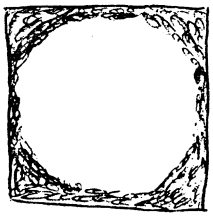
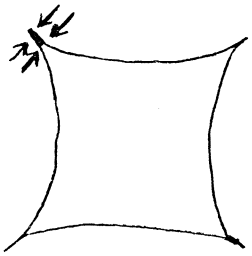
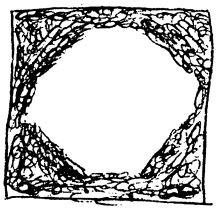
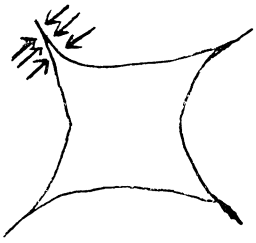
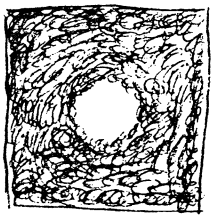
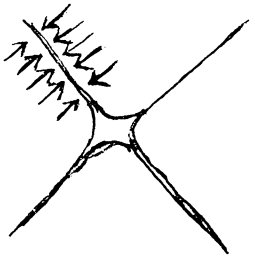
The turtle experiment seems to me to have some significance for a few reasons. First, it seeks to provide an explanation of one part of the wound contraction story which has not yet been explained. The formation of a "stellate scar" from a square wound is used by Abercrombie, Flint and James, in 1954, to argue against certain wound healing hypotheses. They then shrug the phenomenon off by saying, "It is much more probable that the change of shape depends on the relative rigidity of the rim of the wound, which makes the mid-side points more easily pulled inwards than the corner points." And ten years later, James repeated ~~that~~ this phenomenon suggested that, "the wound margins are more readily bent than compressed along their length". In fact, since they then put forth the suggestion that the fibroblasts were responsible for the contraction, I'm not sure how they envisioned the fibroblasts pulling. Apparently it would be a whole sheet pulling together in random orientation with the effect of pulling the whole perimeter toward the center, with the wound margins "bending" under the pressure. This may make some intuitive sense. But it seems to me that even better intuitive sense is made from the idea that

fibroblasts orient themselves and pull as a result of contact and that this contact naturally takes place first between ingrowing fibroblasts in the corners of the wound. These then would pull adjacent rather than opposite edges together about the diagonals.

In the squares in tissue culture the pattern of growth, I believe, does not echo the stellate scar pattern at all. In fact it is theopposite. Instead of cells growing in the stellate pattern

(actually a four-cuspid hypo-cycloid, I think) the grow in the opposite pattern which rounds the edges of the square and finally becomes circular. This would reflect not the shape of the contractin wound, but the pulling direction of the fibroblasts and therefore, continuing the in vitro analogy, the direction of contraction of the wound.

Thus the corners would be stressed as follows:

	in vitro fibroblast growth	wound
(1)		
(2)		
(3)		
(4)		

The second importance of the turtle experiment is to add further evidence to the thesis concerning wound healing where, as James and Taylor put it in 1969, "it appears contraction may be mediated by the fibroblast population of the repair" (I smile at the words "appears" and "may" although I suppose they are the foundation of modern science - and besides this preceded the work of Majno et al. in this area).

Thirdly the turtle experiment has developed a technique which can provide something of an in vitro model for the wound. There is clearly fibroblast outgrowth and pulling which could, perhaps, in some way be applied in vivo.

And lastly and I think most importantly, the experiment has presented a possible technique for studying the fundamental mechanisms of recognition, contact and contraction (demonstrated to exist in the time-lapse film).

On thinking about the film, it seems to me that each of these three areas (recognition, contact, and contraction) are relevant, particularly since this is an invitro study and as such will be better suited for this type of investigation than as a convincing model of the animal."Recognition" of one fibroblast by its neighbor seemed in the film to be non-random, although that may be coincidental. That is, it may be that fibroblast cytoplasm is stretched out only from the directed movement, and any two cells which link and pull do so only because they bumped into eachother. On the other hand it may be that they move purposefully toward eachother. I think more time-lapse studies would quickly clarify this question.

Whether the cells collide or have a rendez-vous, they then form a tight enough junction to support their pulling action. I think this system provides a realistic set of circumstances to look at this junction perhaps by light, EM and fluorescent microscopy. Cells at different stages approaching a junction and forming it could be looked at on a single petri dish (or even a glass

slide) and discrimination between stages could be made with confidence. And continuing on from there, the circumstances involving contraction could also be studied at progressive stages.

This I believe to be the real use of these "squares", since they provide enough of a model that the basic mechanisms, particularly contraction of the fibroblasts, are visible and can be studied.

There is another method which we talked about but never really looked at. That is using outgrowths from explants. One use, of course, would be to remove outgrowing fibroblasts from some part or parts of the tissue (as described above). But there is also another use which I think would be very helpful for studying the contact and contraction phenomena. That is to use two pieces of tissue in close proximity to look at the changes the cells undergo on contact. For instance, from our discussions, it may be that the fibroblasts make their transformation to myo-fibroblasts at a determinable point, perhaps (in vitro) around the time of contact with other fibroblasts. Perhaps a fluorescent study would show the transformation occuring by selectively labelling certain cells (such as those just contacting others). At any rate, I think this would also be a useful method for studying these same areas.

It's nice to end my stay at the Institute by feeling excited. Thank you, really.

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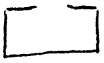
P.S. By the way, Abercrombie, Flint, and James (1956) mention that "smooth muscle cells in tissue culture are indistinguishable from ordinary connective tissue fibroblasts". If they really had smooth muscle cells in tissue culture, it would be crazy not to do the same to compare with the fibroblasts, particularly in response to drugs.

* footnote to page 2 :

The difference was shown when one side of a square was broken thus: 

The ingrowth was several hours ahead in the broken area, and there was not the same orientation in the cells there. Where generally they are parallel to the edges, at the break they were more random, and tending to be perpendicular instead. Actually the orientation is something to be aware of at least. At the edge of the parafilm square, if left there, or the "ditch" if not, the fibroblasts tend to line up. The ditch makes lining up more likely to occur even if the square of parafilm is removed before the culture is enough thickly settled to allow for this orientation. But there is certainly no problem without the "ditches" since the time-lapse films were taken with cells growing on glass coverslips (ie. without ditches).