

Advanced Glycation – New AGE Focus for Diabetes

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The growing epidemic of diabetes will ultimately affect more people than any other disease in the Western World. Over a million adult Australians currently have diabetes and twice that number again are at high risk of developing diabetes in the next five to ten years (1). Diabetes and its associated complications are set to become one of Australia's most costly and significant public health issues. Yet despite the clear and present danger of diabetes, our knowledge of the underlying mechanisms contributing to diabetic pathology remain limited. Clearly, the fundamental abnormality associated with both Type 1 and Type 2 diabetes is the accumulation of circulating sugars (predominantly but not exclusively glucose). But how an excess of sugar contributes to blindness, amputations, kidney failure and cardiovascular disease continues to present an enigmatic challenge to biomedical researchers.

Advanced glycation

Among the irreversible changes that occur as a result of elevated sugars is the formation of advanced glycation end products (AGEs) through a reaction between sugars and the free amino groups on proteins, lipids and nucleic acids. This is known as a Maillard or 'browning' reaction, as similar chemistry is involved in the brown discoloration caused by sugars in heated foodstuffs such as bread, caramel and beer. This reaction requires no enzymatic catalysis but instead depends on temperature, the abundance of reactants and most importantly time. AGE formation occurs only slowly *in vivo* (months to years) meaning that only long-lived molecules such as collagen and lens proteins in the eye are affected. As molecular turnover is reduced with increasing chronological age (2), the amount and variety of AGE-modified tissue increases, contributing to physiological changes that we recognise as signs of ageing (such as cataracts and stiffness). In diabetes, the excess of sugars and increased oxidative stress hasten the browning reaction, meaning that not only are long-lived proteins more heavily modified but also that shorter-lived molecules become targets for advanced glycation (3).

The influence of AGEs on the progress of diabetes complications may best be appreciated by the success AGE inhibitors have had in preventing many of the pathogenic changes of diabetes without reducing the sugar levels (see below) (4). Another reason to suggest that AGEs may be directly damaging comes from the finding that a chronic infusion of AGEs even in non-diabetic animals results in an overproduction of growth factors, enhanced expression of matrix proteins and a pathology resembling that of diabetes (5). AGEs are known to have a wide range of chemical, cellular, and tissue effects (Fig. 1). For example, advanced glycation produces structural and functional alterations of proteins, lipids and nucleic acids through the development of intramolecular and intermolecular cross-linking.

An example of this is the AGE modification of collagen that results in collagen with reduced solubility, flexibility

and a lower breaking point (6). This may be readily appreciated by the comparative toughness of old sheep meat compared to that of tender lamb. AGE-modified collagen is also more resistant to enzymatic digestion contributing to collagen accumulation as much as to its culinary value. In ageing and diabetes, these changes contribute to increased vascular and cardiac stiffness (7).

Normally, molecules in the vessel wall slide freely over one another, permitting vessel dilation and contraction with each heart beat. However, cross-linked proteins transform the vessel into a stiffened, inelastic tube putting increased stress on the vessel wall that literally results in hardening of the arteries and hypertension. AGE cross-linked proteins also act as a sticky web, causing passing macromolecules to become irreversibly trapped. In diabetes this results in harmful cholesterol becoming stuck in the vessel wall contributing to atherosclerosis. AGE modification of receptor proteins may also alter ligand binding and signalling pathways important for normal function. For example, glycation of the low-density lipoprotein (LDL) receptor results in impaired lipid uptake, also contributing to cholesterol accumulation in diabetic patients (3).

AGE receptors

Many of the effects of AGEs may also be mediated by interaction with specific receptors and binding proteins. These receptors are present on various cell types, including macrophages, endothelial cells, and smooth muscle and renal cells, as well as nerve cells. The role of these receptors is still unclear. One thought is that they recognise senescent proteins for excretion or catabolism (3,8). The degree of AGE-modification therefore represents a mechanism to judge the age of a molecule (i.e. old cross-linked collagen is preferentially catabolised to newly laid down supple proteins). The interaction of AGEs with their receptors also results in activation of a number of transcription factors including nuclear factor kappa B (NF- κ B), release of pro-inflammatory cytokines and expression of growth factors and adhesion molecules implicated in the pathogenesis of the complications of diabetes (3). Curiously, the promoter region of one of the AGE receptor genes also contains NF- κ B binding sites, producing a self-perpetuating pathway whereby increased AGEs lead to increased expression of AGE-receptors and therein increased AGE receptor signalling (9). This vicious cycle occurs particularly at sites of diabetes-associated injury (kidney, blood vessels, and retina) (10).

One of the most interesting effects of AGEs appears to be to influence the differentiation of cells. For example, the application of AGEs onto kidney tubular cells is able to change the growth pattern of these cells into a myofibroblast lineage (Fig. 2) (11). This process, called transdifferentiation, may be important in the development of fibrosis in diabetic kidney disease as myofibroblasts produce large amounts of collagen and connective tissue.

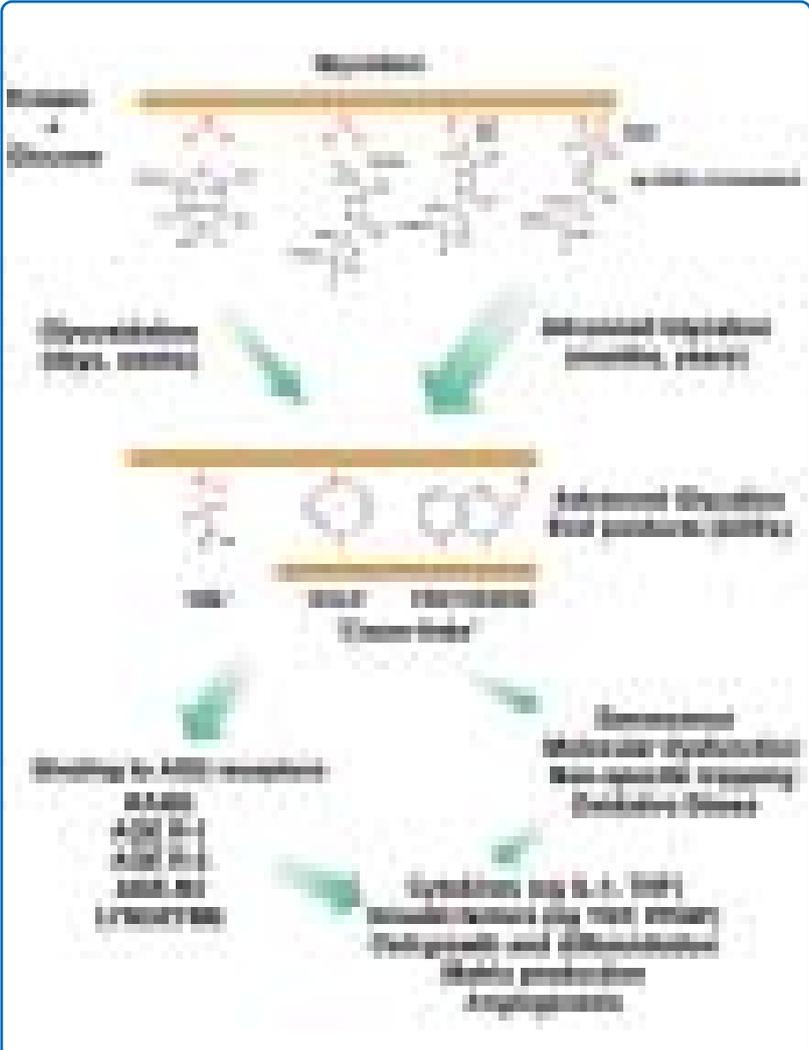


Fig. 1. The formation and effects of advanced glycation end products (AGEs).

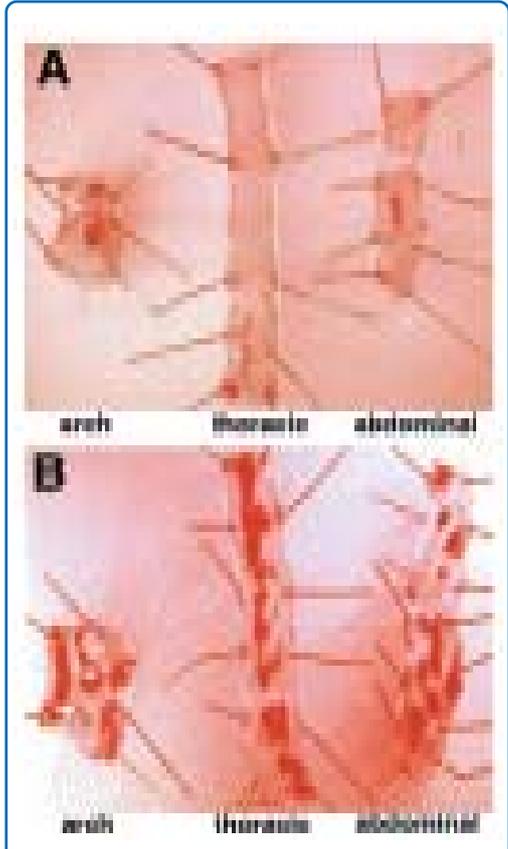


Fig. 3. Atherosclerotic lesions in the aorta. Atherosclerosis in ApoE knockout mice without diabetes (A) is much less than that in animals with diabetes (B). Sudan red staining yields a deep red colour indicating atherosclerotic lesions; the pink background stain represents normal aortic tissue. Atherosclerosis is predominantly found in thoracic and abdominal aortic regions. Pins hold the aortic preparations in place.



Fig. 2. Differentiation of cells induced by AGEs. Normal rat kidney (NRK 52E) tubular epithelial cells trans-differentiate from a classic cobblestone morphology seen (A) into elongated myofibroblasts which stain for α -smooth muscle actin (B). A trans-differentiated cell (arrow) can also be seen in a human kidney with diabetes (C) (11). Cell preparations are counterstained with haematoxylin. The magnification in C is twice that in A and B.

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This effect can be completely prevented by blocking the receptor for AGEs, implying a crucial role for AGE receptors in cell differentiation. In addition to the known receptors, novel AGE-binding sites are continuing to be discovered. It seems very likely that new interactions between AGEs and normal physiology will emerge, and contribute to our understanding of both ageing and diabetes.

AGE and oxidative stress

Reactive oxygen species (ROS) – better known as free-radicals – have long been associated with the damaging effects of ageing. Oxidative stress that leads to the formation of ROS is also recognised as a key component in the development of diabetic complications. Our studies have demonstrated that oxidative stress is increased in diabetic animals in proportion to the quantity of AGE accumulation (12). Oxidative stress can augment the formation of AGEs through so-called glycoxidation. However, AGEs can also lead to enhanced formation of free radicals both through binding of AGEs to their receptors and directly through catalytic sites in the structure (13).

New approaches to AGEs

Recent attention has focused on methods to decrease AGEs in patients with diabetes. The first drug to be used, aminoguanidine, succeeded in reducing AGEs and preventing injury. However, the actions of this drug are not confined to the inhibition of AGE formation and overall it has proved too toxic in the clinical setting. Newer agents such as ALT-946 (N-(2-Acetamidoethyl)-hydrozine-carboximidamide hydrochloride) may be more specific and have fewer side effects. Our group has already shown beneficial effects of ALT-946 on renal AGE accumulation, albuminuria and glomerular filtration rate in a model of experimental diabetes (14).

ALT-711 (4,5-Dimethyl-3-(2-oxo-2-phenylethyl)-thiazolium chloride) is the first of a new generation of compounds for clinical use, able to cleave pre-formed AGE cross-links. These cross-link breakers have been shown to be capable of reversing AGE-mediated vascular stiffness and distensibility in diabetic rats (8). We have demonstrated that this agent is associated with reduced serum AGE levels, tissue AGE accumulation and improved kidney functions (4). If ALT-711 proves equally effective in humans, such agents may prove highly beneficial in the reversal of AGE-related disease.

Some of the treatments already used to prevent diabetic complications may also act through AGE-dependent mechanisms. For example, it appears that angiotensin converting enzyme (ACE) inhibition prevents an increase in both tissue and serum AGEs levels in animals with experimental diabetes (15). The major site of this action by ACE inhibitors is yet to be established. The formation and accumulation of AGEs in diabetes is clearly multi-factorial involving not only chronic high sugars but also oxidative pathways and processes linked to AGE degradation and removal. It is possible that ACE inhibitors act through one or many of these mechanisms.

One of the most important interventions in limiting the accumulation of AGEs remains the prevention of progression of diabetic kidney disease. The risk of complications including vascular events and death are increased markedly with the degree of kidney damage in

diabetes. As renal clearance may be the most important mechanism for removal of AGEs from the body, this progressive decline in renal function leads to an accelerated accumulation of tissue AGEs and produces the pattern of accelerated tissue injury so frequently seen in advanced diabetes. It follows that new methods to maintain or augment the removal of AGEs from the body may prove to be of considerable value in the successful management of diabetes.

AGEs and genomics

New tools for molecular analysis are also providing important insights into the mechanisms by which AGEs may produce organ injury. It is clear that AGEs interact with a number of pathways and many of these pathways are not exclusive, meaning that a variety of noxious stimuli can produce similar effects or augment the effect of other insults. A clear understanding of which pathways are critical – and moreover which steps may be amenable to modification without disrupting normal physiology – will be vital in the development of new therapeutic initiatives.

Recent advances in the production of cDNA microarrays have made it possible to monitor the expression of thousands of genes simultaneously and hence provide an important tool for this puzzle. Gene arrays enable a snapshot to be taken of the expression status of every gene at any point in time. The technique is best described as a comparative hybridisation experiment in which RNA is extracted from control and test samples. Labelled cDNA is produced and hybridised to a gene chip containing thousands of immobilised cDNAs or oligonucleotides. A comparison of the extent of hybridisation of each sample to the same cDNA spot then allows the expression level of each gene in the two samples to be compared. The usual aim of this approach is to identify key genes or at least likely candidates in any disease process.

Gene arrays can also be used to identify patterns of change in gene expression (following treatment, for example, with AGEs) and hence can aid better definition of diseases in terms of gene expression, clinical features and outcome. For example, we have used the gene array procedure in an animal model to identify a number of genes that are altered in the diabetic kidney. We are currently using it to study the effects of AGEs in both *in vivo* and *in vitro* model systems to help us better understand the signalling pathways involved. In preliminary experiments, AGEs appear to activate a number of transcription factors and signalling molecules involved in processes like transdifferentiation (11). Further work is being carried out to confirm the changes and better understand the biochemistry of these molecules and the processes involved in the development of disease.

Regardless of the amount of information obtained by such experiments, the nature of the questions that can be answered by gene profiling is limited. There has been a massive explosion of genetic information resulting from the Human Genome Project and profiling experiments, but we are finding that understanding the biochemical significance of gene expression changes has moved us back into the realm of proteomics, the study of cellular proteins. The simple reason is that genetic information is static while the protein complement of the cell is dynamic. Genomic information does not predict post-translational

modifications (of which browning is an important one), the amount of gene product made, the timing of its translation, alternate splicing or events involving multiple genes (such as ageing, stress, diabetes). Genomics and proteomics are therefore essentially complementary disciplines, each providing pieces to a larger puzzle. Thus it is useful to employ both gene profiling to determine the transcription of which genes have been activated as well as proteomics to define the signalling pathways that mediate the transmission of signal to the nucleus to drive transcription. Using this combined approach we are studying the effects of AGEs in cell line models to understand the signalling pathways activated when cells are exposed to AGEs, hoping to identify genes that are undergoing post-translational changes prior to any changes in gene expression, as well as newly transcribed genes.

Models of atherogenesis

Eight out of every ten people with Type 2 diabetes will die from cardiovascular disease (CVD). While classical risk factors for CVD, such as smoking, cholesterol and hypertension operate in patients both with and without diabetes, the absolute risk of death is two to four times greater in patients with diabetes and progressively greater with each additional risk factor (16). In diabetes, as with ageing, blood vessels become increasingly inelastic and narrowed with accumulation of cholesterol and protein in the vessel wall. As a direct consequence, recent research has focussed on understanding the mechanisms by which AGEs can augment this process.

Research by our group has demonstrated AGEs within atherosclerotic lesions in both extracellular and intracellular locations correlating with the size and complexity of the lesions (17). Expression of the adhesion molecules, considered a hallmark of early atherosclerosis, is induced by the AGEs interaction (18). Endothelial cells lining blood vessels may also react to AGEs to promote cell adhesion, inflammation and the formation of blood clots (19). In addition, AGEs interfere with the synthesis and action of nitric oxide (NO), the most important dilating/relaxing molecule in the vessel wall (3). These alterations may also contribute to the impaired relaxation of blood vessels seen in patients with diabetes leading to hypertension and impotence.

Like diabetes, atherosclerosis occurs over years to decades. One of the major stumbling blocks in diabetic research has been the lack of development of appropriate experimental models of accelerated atherosclerosis that enable the process to be studied within the working lifetime of a researcher. In addition, the induction of experimental diabetes in rats or rabbits paradoxically results in less rather than more arterial disease. However, recent work has led to the development of an *in vivo* model of progressive diabetic renal disease in association with atherosclerosis using knockout mice in whom the gene for Apolipoprotein E (ApoE) has been genetically deleted. Absence of this gene in mice results in increased cholesterol levels and advanced atherosclerotic lesions by the age of 30 weeks. However with the induction of diabetes, complex lesions are present in the aorta as early as six weeks (Fig. 3).

This model allows us to examine the role of AGEs in atherosclerotic disease. Reduction of AGEs with the AGE cross-link breaker (ALT-711) or aminoguanidine results in a significant reduction in vascular stiffening and

amelioration of various pathologic changes including a reduction in plaque size, matrix accumulation and vascular narrowing (20). Importantly, blockade of the renin-angiotensin system using ACE inhibitors can also attenuate diabetes-associated atherosclerosis in this model (17).

A new AGE focus for diabetes

Increased longevity brings an increased incidence of a variety of diseases. Apart from diabetes, the accumulation of AGEs is thought to contribute to the tissue damage in a variety of conditions as diverse as atherosclerosis and Alzheimer's disease. Glycation is not the only mechanism by which excess sugars may produce damage. Nonetheless, AGEs appear to be capable of contributing to diabetic pathology both on their own and in combination with other pathways (Fig. 3). Research in the next decade will likely throw new light on how AGEs interact as part of the normal physiology of senescence and accumulate in disease states. None of us is getting any younger and if, as it is predicted, a quarter of those reading this article will develop diabetes, it seems evident that AGEs should represent an important research focus for the future.

References

- Zimmet, P., *et al.* (2001) Diabetes and associated disorders in Australia. International Diabetes Institute (see also article by Zimmet and Chisholm in this *Showcase on Research*)
- Szweda, P.A., Friguet, B., and Szweda, L.I. (2002) *Free Radic. Biol. Med.* **33**, 29-36
- Vlassara, H., and Palace, M.R. (2002) *J. Intern. Med.* **251**, 87-101
- Cooper, M.E., Thallas, V., Forbes, J., Scalbert, E., Sastra, S., Darby, I., and Soulis, T. (2000) *Diabetologia* **43**, 660-664
- Vlassara, H., Striker, L.J., Teichberg, S., Fuh, H., Li, Y.M., and Steffes, M. (1994) *Proc. Nat. Acad. Sci. USA* **91**, 11704-11708
- Fu, M.X., Wells-Knecht, K.J., Blackledge, J.A., Lyons, T.J., Thorpe, S.R., and Baynes, J.W. (1994) *Diabetes* **43**, 676-683
- Vaitkevicius, P.V., Lane, M., Spurgeon, H., Ingram, D.K., Roth, G.S., Egan, J.J., Vasani, S., Wagle, D.R., Ulrich, P., Brines, M., Wuerth, J.P., Cerami, A., and Lakatta, E.G. (2001) *Proc. Nat. Acad. Sci. USA* **98**, 1171-1175
- Neeper, M., Schmidt, A.M., Brett, J., Yan, S.D., Wang, F., Pan, Y.C., Elliston, K., Stern, D., and Shaw, A. (1992) *J. Biol. Chem.* **267**, 14998-15004
- Li, J.F., and Schmidt, A.M. (1997) *J. Biol. Chem.* **272**, 16498-16506
- Soulis, T., Thallas, V., Youssef, S., Gilbert, R.E., McWilliam, B., Murray-McIntosh, R.P., Cooper, M.E. (1997) *Diabetologia* **40**, 619-628
- Oldfield, M.D., Bach, L.A., Forbes, J.M., Nikolic-Paterson, D., McRobert, A., Thallas, V., Atkins, R.C., Osicka, T., Jerums, G., and Cooper, M.E. (2001) *J. Clin. Invest.* **108**, 1853-1863
- Cooper, M.E. (2001) *Diabetologia* **44**, 1957-1972
- Yim, M.B., Yim, H.S., Lee, C., Kang, S.O., and Chock, P.B. (2001) *Ann. NY Acad. Sci.* **928**, 48-53
- Forbes, J.M., Soulis, T., Thallas, V., Panagiotopoulos, S., Long, D.M., Vasani, S., Wagle, D., Jerums, G., and Cooper, M.E. (2001) *Diabetologia* **44**, 108-114
- Forbes, J.M., *et al.* *Diabetologia* (in press)
- Cooper, M.E., and McNally, P.G. (1998) In *The kidney and hypertension in diabetes mellitus*. Mogensen, C.E., editor. Kluwer, Boston 419-432
- Candido, R., Jandeleit-Dahm, K.A., Cao, Z., Nesteroff, S.P., Burns, W.C., Twigg, S.M., Dilley, R.J., Cooper, M.E., and Allen, T.J. (2002) *Circulation* **106**, 246-253
- Schmidt, A.M., Hori, O., Chen, J.X., Li, J.F., Crandall, J., Zhang, J., Cao, R., Yan, S.D., Brett, J., and Stern, D. (1995) *J. Clin. Invest.* **96**, 1395-1403
- Vlassara, H., Fuh, H., Donnelly, T., and Cybulsky, M. (1995) *Mol. Med.* **1**, 447-456
- Asif, M., Egan, J., Vasani, S., Jyothirmayi, G.N., Masurekar, M.R., Lopez, S., Williams, C., Torres, R.L., Wagle, D., Ulrich, P., Cerami, A., Brines, M., and Regan, T.J. (2000) *Proc. Nat. Acad. Sci. USA* **97**, 2809-2813

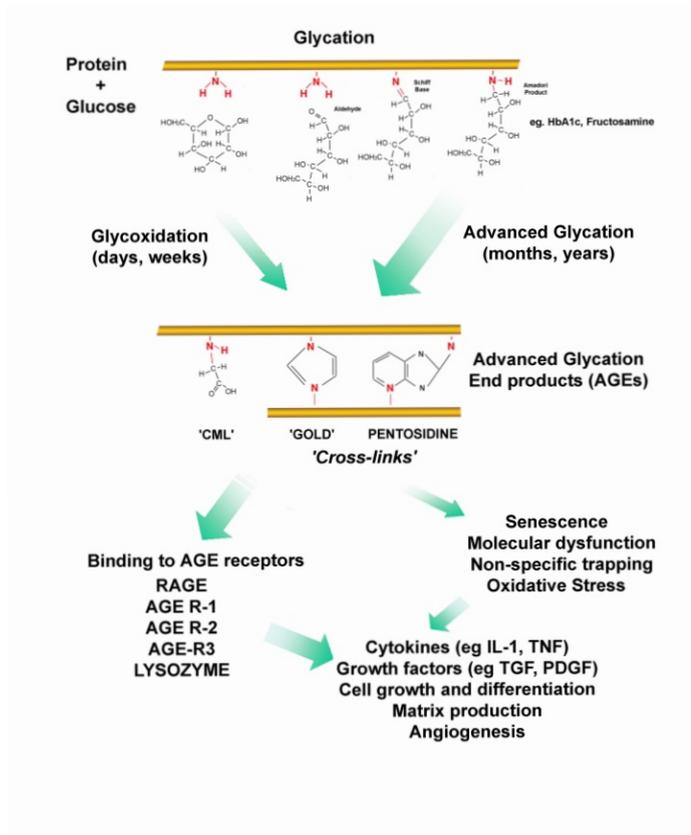


Fig. 1

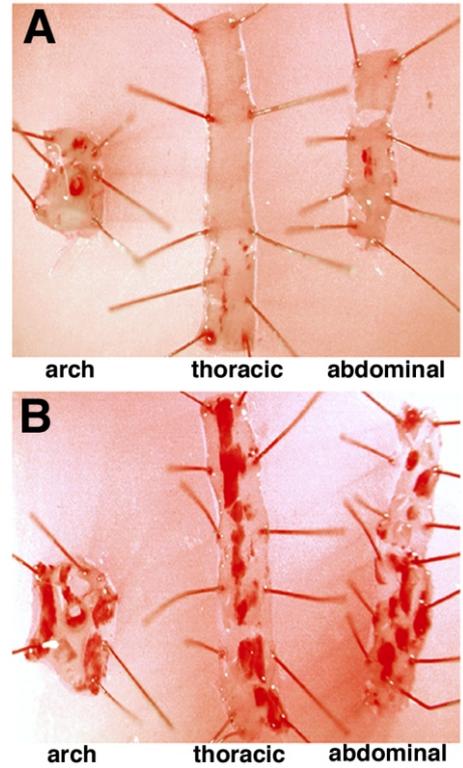


Fig. 3

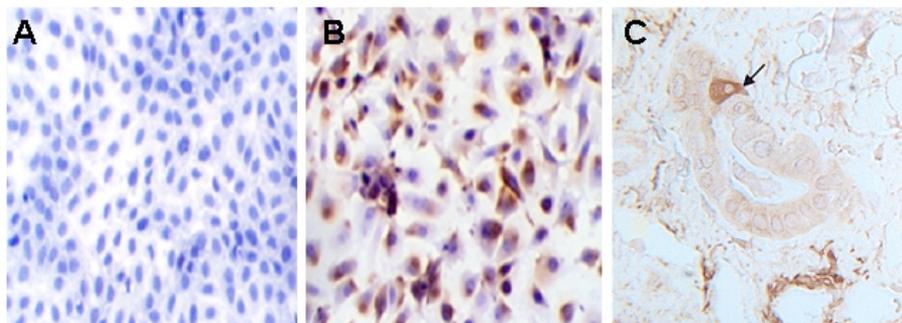


Fig. 2