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(54) Title: DEVICE FOR PROMOTING ENDOTHELIAL CELL MOTILITY AND/OR VASCULARISATION

(57) Abstract

A device for promoting endothelial cell motility and/or vascularisation in man and other vertebrate animals which comprises a carrier such as a sheet or tube of a plastic material, which carrier is impregnated or coated with a source of copper I, copper II or tin II ions such that when applied to or inserted into an animal the ions can diffuse into the surrounding tissue of the animal. The device can be used to promote vascularisation after surgery or on parts of the body lacking adequate vascularisation such as skin ulcers. It can be used in the form of a tube for arterial by-pass surgery wherein the increased motility of the endothelial cells causes faster carbonisation of the interior of the tube and thereby lessens the risk of blood clots forming during such surgical procedures.

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DEVICE FOR PROMOTING ENDOTHELIAL CELL MOTILITY AND/OR VASCULARISATION

DESCRIPTION

TECHNICAL FIELD

The present invention relates to a device for application to or implantation into the body of a vertebrate animal, including man, for the purpose of increasing endothelial cell motility in the subject or vascularisation of the subject in the region of the device.

BACKGROUND ART

The heart blood vessels of vertebrate animals are lined with a single layer of smooth flattened cells known as endothelial cells.

It has previously been observed that extracts from certain cells, such as Walker carcinoma cells, have the ability to promote vascularisation of vertebrate animals. These extracts were generally unidentified compounds of high molecular weight. The vascularisation takes the form of the development of a dense bed of blood vessels in the area in which the extract is applied to the subject animal, or throughout the animal if the extract was applied systemically as by subcutaneous injection.

It has been postulated that the vascularisation results from a chemotactic response of endothelial cells to these extracts which is associated with the ability of the extract to increase the motility of endothelial cells.

One of the techniques used to test the vascularisation ability of a compound is to incorporate the extract into a bead of a synthetic plastics material from which it could diffuse and to implant the pellet in the anterior eye chamber of a test animal. Vascularisation activity was shown by the growth of capillaries into the anterior eye chamber of the animal. If the extract had no vascularisation ability there is no growth of capillaries towards the pellet and into the eye.

DISCLOSURE OF INVENTION

The present inventors have discovered that certain inorganic metal ions have the ability to promote vascularisation and they have further found that this vascularisation activity goes hand in hand with the ability of these metal ions to increase endothelial cell motility i.e. the extent of movement exhibited by the cells.

The present invention consists of a device for promoting endothelial cell motility and/or vascularisation in vertebrate animals including man comprising a carrier applicable to or insertable into the body of a vertebrate animal, characterised in that the carrier contains, or is coated with, a source of copper I, copper II or tin II ions and in that the ions can diffuse from the carrier into the surrounding tissues of an animal to which the carrier is applied or into which it has been inserted.

The device according to this invention can be used in two ways. The first utilises the property of increasing the motility of endothelial cells without promoting vascularisation while the second utilises the property of increased cell motility to bring about a desired vascularisation.

An example of the first use of a device according to the present invention is a device in the form of a tube which can be used in arterial by-pass surgery. A problem encountered with such surgery in the past has been the danger of the development of blood clots within the artifical artery before the interior of the tube has been colonised by endothelial cells which have the effect of preventing platelet adhesion, and the formation of blood clots, in natural blood vessels. The use as the artificial artery of a device according to this invention increases the general motility of the

endothelial cells in the animal and by chemotactic reaction causes them to migrate towards the device to thereby colonise the interior of the tube more rpaidly than would normally be the case and thereby reducing the risk of blood clots developing in the patient.

Examples of the second way in which a device according to the present invention can be used include the use of a device in the form of a sheet which can be laid over skin ulcers to promote revascularisation of the ulcerated skin; the use of a device in the form of absorbable sutures which promote revascularisation of the site of a surgical incision; and the use of a device in the form of a cover or housing for a prosthetic device such as a heart pacemaker to promote vascularisation and tissue growth in the region in which the prosthetic device has been implanted.

The carrier is preferably a synthetic plastics material, however, other carriers could be used particularly adsorbable materials such as collagen or albumin whether in a natural or reconstituted form. The most preferred material for the formation of the carrier is an ethylene vinyl acetate copolymer described by Longer R. and Folkman J. in Nature 263 (1976) 797. The carrier may be of any suitable physical shape such as a tube, thread, sheet or a net or web. Alternatively the carrier may be a cover or housing for a prosthetic device or the prosthetic device itself. If the metal ions are disposed on the carrier as a coating, as opposed to being disposed within the carrier and diffusable therefrom, a wider variety of materials could be used for the carrier itself. In this case the coating would have to be non-toxic and to be capable of releasing the metal ions at the desired rate.

It is preferable that the carrier contain the metal

ions in a concentration of from 1 x 10^{-7} to 1 x 10^{-2} molar, preferably from 1 x 10^{-6} to 5 x 10^{-4} molar. The source of the metal ions may be any suitable non-toxic salt or complex of the metal. Simple salts such as copper II chloride, copper II sulphate, copper II phosphate, copper I chloride, copper II nitrate, and tin II sulphate, tin II chloride may be used as well as complex ions which contain the desired metallic cation. Organo-metallic salts or complexes may also be used in the present invention. These include copper I, copper II and tin II salts or complexes with lactic acid, histidine, succinic acid, nor-adrenaline, amino acids, penacillamine, riboflavin and formic acid.

BEST MODE OF CARRYING OUT THE INVENTION

The following description is illustrative of the best method of carrying out the present invention without being limitative on the broad scope of the following claims:-

(a) determination of endothelial stimulating activity of metal ions.

(i) Materials and Methods

Cells. A line of bovine aortal endothelial cells, BAE, established without recourse to any added growth factors has been described in McAuslan, B.R., Reilly, W. and Hannan, G.N. J. Cell Phsiol 100 (1979) 87. This line was maintained in medium 199 by additional folate and supplemented with 10% fetal calf serum.

A variant of these cells, BAE.VI, whose distinguishing characteristic is lack of contact inhibition, was isolated and a line established as described in the abovementioned article.

Estimation of Motility. Motility was demonstrated by the procedure of Albrecht-Buehler

(Albrecht-Buehler, G. Cell II (1977) 395). The tracks formed as motile cells ingested gold particles, were visualised by low power (x20 - x40) light microscopy under dark field illumination. It was found that the following minor details are imperative for application of the technique to endothelial cells:

(i) The colloidal form of gold and the thickness of the gold film must be controlled. To ensure this, the formalin used as a precipitatnt must be prepared freshly prior to use. The collidal gold solution should then appear blue to transmitted light; a purple coloured solsution, indicating a different particle size, yields poor results.

(ii) To 18 mm diameter coverslips coated with albumin we apply only 0.25 ml of gold coating mixture. After incubation, excess fluid is removed by aspiration and while still wet, the coverslips are dipped into medium to remove free gold particles. Other than these small modifications, the procedure of Albrecht-Buehler was followed in detail.

Usually 10 single cell tracks on each of four fields was measured for each coverslip and three coverslips for each test system were screened. Values for track lengths given are the mean of the sum of approximately 80-100 individual tracks. BAE cells when not stimulated always showed, around each cell, a small clear zone, the diameter of which we have given as a track length.

To establish cultures for testing, cells were plated at a density of 10^4 cells/6 cm dish containing gold-coated coverslips. For easier estimation of track length, cells were sometimes plated at 2-5 x

10^{-3} cells/6 cm dish. This reduced the incidence of crossed or fused tracks. To replicate plates, medium 199 containing 10% fetal calf serum (5 ml) was added and after cells had attached, solutions of salts to be tested (not more than 0.1 ml) were introduced. Coverslips were examined for tracks 24 to 72 hours later. Track length was estimated electroncially by a precalibrated Summagraph Digitizor (Anderson Digital Products, Australia). Results are expressed as a mean value ± a standard deviation (S.D.) of 30%.

EDTA Treated Parotid Extract. Crude bovine parotid aqueous extract (4) was adjusted to 10^{-5}M sodium ethylene diamine tetracetate, EDTA. The mixture (2 ml) was applied to a column of Sephadex G10 (0.9 x 10 cm) and developed with water. The excluded fraction was collected for testing. As a control, extract not treated with EDTA was otherwise treated identically.

Isolectric Focusing. Ceruloplasmin type III (3 ml, 150 mg protein) was subjected to preparative isolectric focusing (35 W constant power) in a pH5-9 gradient of LKB ampholynes using the Radola technique. The region of angiogenic activity (isolectric point 7.8) as determined by renal vascularisation assay was eluted with water and the eluate freeze-concentrated to the quivalent of the original concentration at which applied.

Reagents. Ceruloplasmin (human, type III) was purchased from Sigma (U.S.A.). All metal salts tested were analytical grade reagents.

(ii) Results

The above materials and methods were used to demonstrate that certain metal ions mobilise cultured endothelial cells. These are obvious

practical difficulties in establishing a gradient of ions in liquid culture medium used to grow cells on a monolayer. Instead measurements were made of the "random walk" migration of cells in response to the direct addition of the appropriate metal salt to the growth medium.

Culture bovine aortal endothelial cells, BAE showed negligible motility on gold-coated coverlips. When either $CuCl_2$ or $CuSO_4$ was added to the medium final concentrations 10^{-6}M) cell motility was markedly stimulated. Neither Zn, as its divalent chloride or sulphate, nor a variety of salts of Mo^{II}, Al^{III}, Cr^{II}, Co^{II}, Mn^{II}, Fe^{III} over the range of concentrations tested (10^{-7}M to 10^{-4}M) altered cell motility. However, $SnCl_2$ (10^{-6}M) did stimulate cell motility quite markedly. By quantitative motility we estimated the optimal level of added copper or tin to be approximately 2 x 10^{-6}M, however, a cell response could be detected at concentrations of added copper or tin salts of 10^{-7}M. In control cultures, occasionally some cells were found to be motile but these constituted less than 5-10% of the total population.

In test cultures adjusted to optimal concentrations of copper or tin, at least 95% of the cells responded.

There is specificity in the response of endothelial cells since we found that neither bovine aortal fibroblasts, bovine aortal smooth muscle cells nor bovine kidney cells migrated in response to added copper or tin ions.

Using both BAE and BAE.VI cells, the present inventors tested other valence states of copper and tin in the form of cuprous chloride and stannic chloride. Cu^{I} ions enhanced the motility of both

BAE and BAE.VI cells whereas Sn^{IV} ions were inactive.

Ceruloplasmin. Since copper is the active principle of bovine angiogenic factor and cultured tumor cells have high angiogenic factor activity such cells must utilise or modify exogeneous copper. The likely major source of copper in culture medium is the serum cuproprotein ceruloplasmin which contains 8 moles of copper/mole protein. The ceruloplasmin in endothelial cell culture medium is probably at too low a concentration to be effective, but when purified ceruloplasmin (30 ng ceruloplasmin/ml) was added to medium at a level equivalent to approximately 10^{-6}M copper it caused a marked increase in the motility of cells. It was found that the response of BAE cells to ceruloplasmin is highly variable in that they sometimes do not respond. However, over numerous experiments BAE.VI cells are consistently and markedly mobilised by ceruloplasmin. When human ceruloplasmin was subjected to isolectric focusing (see Methods) angiogenic activity as determined by renal assay was located at regions corresponding to isolectric points of 4.5 (uncharged ceruloplasmin) and at a region of isolectric point 7.8. When the latter material was eluted from the isofocusing bed and tested on BAE.VI cells (at a concetration equivalent og 30 ng of unfocused ceruloplasmin per ml) it consistently caused a pronounced increase in cell motility.

Motility as an Assay for Neovascularising (Angiogenic) Activity. Two bioassays used for detection of fractions thast cause new capillary formation are pellet vascularisation in the anterior eye chamber or neovascularisation of the renal

medulla and cortex (McAuslan, B.R. and Hoffman, H. Exp. Cell. Res. 119 (1979) 181.) These assays are time consuming and suffer other obvious drawbacks asociated with whole animal testing. Furthermore, the reliability of these assays in our experience, sometimes decreases when very low molecular weight angiogenic substances are tested. Studies on the fractionation of cells and tissues have shown that neovascularising activity was associated with substances of molecular weight 80-100,000, 3,000 and 210. If copper is indeed the active component in each of these fractions, it was of interest to see if these were positive in the cell motility assay. The results can be summarised as follows:

(i) cell mobilising activity and neovascularising activity coinicde with fractions (M.W. 80-100,000, 3,000 and 210 respectively) of Walker carcinoma extract. Fractions that were not angiogenic did not enhance motility.

(ii) Crude bovine parotid gland extract or sub-fractions of molecular weight 80,000, 3,000 and 210 (1,5) promote neovascularisation (1,5). Only these fractions promoted cell motility. In accord with the contention that cell motility is due to metal ions, cell mobilising activity in crude parotid extract was eliminated by EDTA peretreatment and restored by readdition of copper ions.

(b) Determination of vascularisation activity of devices according to this invention.

EXAMPLE I

Solutions containing $(Cu\ Cl_2)^{-2}$ at a concentration of 10^{-5} molar were incorporated into sterile polymer pellets of ethylene-vinyl acetate copolymer by the technique described in Langer, R.

and Folkman, J. Nature 263 (1976) 797. These beads were inserted into the anterior eye chamber of test rats after the procedure of Gimbrone, M.A., Leapman, S., Cotran, R.S. and Folkman, J., Journal of the National Cancer Institute 50 (1973) 219.

A striking outgrowth of capillaries from the iris towards the pellet of copper (II) ion containing polymer was observed reproducibly.

EXAMPLE II

Example II was repeated using pellets incorporating concentrations of copper (II) ion derived from copper sulphate of from 10^{-5} to 10^{-3} molar.

In each case the striking outgrowth of capillaries from the iris towards the pellet was observed. There was no observable difference in the rate of growth of the capillaries with increasing copper (II) ion concentration.

CLAIMS:

1. A device for promoting endothelial cell motility and/or vascularisation in vertebrate animals, including man, comprising a carrier applicable to or insertable into the body of a vertebrate animal, characterised in that the carrier contains or is coated with a source of copper I, copper II or tin II ions and in that the ions can diffuse from the carrier into the surrounding tissues of an animal to which the carrier is applied or into which it has been inserted.

2. A device as claimed in claim 1 in which the carrier is made of a synthetic plastics material.

3. A device as claimed in claim 2 in which the carrier is made of a copolymer of ethylene and vinyl acetate.

4. A device as claimed in claim 1 in which the carrier is formed of collagen or another adsorbable material.

5. A device as claimed in claim 1 in which the carrier contains the metal ions in a concentration of from 1 x 10^{-7} to 1 x 10^{-2} molar, preferably from 1 x 10^{-6} to 5 x 10^{-4} molar.

6. A device as claimed in claim 1 in which the carrier is in the form of a tube, sheet, thread or net.

7. A device as claimed in claim 1 in which the carrier forms a prosthetic device or a housing or cover for such a prosthetic device.

INTERNATIONAL SEARCH REPORT

International Application No PCT/AU80/00007

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all) [3]

According to International Patent Classification (IPC) or to both National Classification and IPC

Int. Cl.[3] A61M 37/00, A61K 31/30, 31/32, 31/555, 33/24, 33/34, A61F 1/00, A61L 15/03, 17/00

II. FIELDS SEARCHED

Minimum Documentation Searched [4]

Classification System	Classification Symbols
IPC	A61M 37/00, A61K 31/30, 31/32, 31/555, 33/24, 33/34,
US. Cl.	128/260; 424/131, 140, 142, 143, 288, 294

Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched [5]

AU:IPC as above; Australian Classification 87.18, 87.40, 87.44

III. DOCUMENTS CONSIDERED TO BE RELEVANT [14]

Category *	Citation of Document, [16] with indication, where appropriate, of the relevant passages [17]	Relevant to Claim No. [18]
X	AU, B, 9914/55 (202914), published 1955 December 22, see p3, Simba AG.	1-7
X	AU, B, 18221/62 (261796), published 1963 December 5, Glaxo Laboratories Ltd.	1-7
X	AU, B, 34614/63 (274375), published 1965 March 4, see p9, Dow Corning Corp.	1-7
X	AU, B, 21311/67 (409861), published 1968 November 7, see p6, Ceskoslovenska Akademie Ved.	1-7
X	AU, B, 14562/70 (456708), published 1971 November 4, NV Philips Gloeilampenfabrieken.	1-7
X	AU, B, 38834/72 (477441), published 1973 August 16, see p4, Rhone-Poulenc S.A.	1-7
X	AU, B, 11226/76 (497446), published 1977 September 8, see pp7, 9, ICI Australia Ltd.	1-7
P	AU, A, 41516/78, published 1979 June 7, ICI Australia Ltd.	1-7
P	AU, A, 46497/79, published 1979 November 15, V.A. Garten.	1-7
X	US, A, 1470422, published 1923 October 9, Bowlus.	1-7
X	US, A, 2275292, published 1942 March 3, Edmondson.	1-7
X	US, A, 2719811, published 1955 October 4, see col 1, l 62-65, col 2, l 19-20, 33, col 4, l 36-39, Cook.	1-7
X	US, A, 3932638, published 1976 January 13, see col 2, l 51, col 3, l 22, col 8, l 49-52, col 9, l 44, Coirre.	1-7

* Special categories of cited documents: [16]

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"X" document of particular relevance

IV. CERTIFICATION

Date of the Actual Completion of the International Search [2]	Date of Mailing of this International Search Report [2]
11 JULY 1980	16 JULY 1980 (16.07.80)
International Searching Authority [1]	**Signature of Authorized Officer [20]**
Australian Patent Office	A S MOORE A.A. Moore

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FURTHER INFORMATION CONTINUED FROM THE SECOND SHEET

X	US, A, 4123511, published 1978 October 31, Heintze.	1-7
X	Chemical Abstracts, Volume 78, No. 5 Issued 1973, Feb 5 (Columbus, Ohio, USA) E. Gileadi et al, "Antithrombogenic Characteristics of Cathode Polarised Copper Prostheses", Abstract No. 24719, J. Biomed. Mater. Research, 1972, 6(6), 489-97, (Eng).	1-7
X	Chemical Abstracts, Volume 85, No. 25 Issued 1976, Dec 20 (Columbus Ohio, USA), F. Bischoff et al, "Toxicologic Studies of Tin Needles at the Intrathoracic Site of Mice", Abstract No. 187418, Res. Comm. Chem. Pathol. Pharmacol., 1976, October, 15(2), 331-40 (Eng).	1-7
X	Chemical Abstracts, Volume 88, No. 7 Issued 1978, Feb 13 (Columbus Ohio USA), W R Walker, "The Copper Bracelet Story: Some Aspects of the Role of Copper in Inflammatory Disease", page 59, col 2, Abstract No. 45156, Chem. Aust., 1977, 44(10), 247-52, (Eng)	1-7

V. ☐ OBSERVATIONS WHERE CERTAIN CLAIMS WERE FOUND UNSEARCHABLE [10]

This international search report has not been established in respect of certain claims under Article 17(2) (a) for the following reasons:

1. ☐ Claim numbers ....., because they relate to subject matter [12] not required to be searched by this Authority, namely:

2. ☐ Claim numbers ....., because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out [13], specifically:

VI. ☐ OBSERVATIONS WHERE UNITY OF INVENTION IS LACKING [11]

This International Searching Authority found multiple inventions in this international application as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims of the international application.

2. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims of the international application for which fees were paid, specifically claims:

3. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claim numbers:

Remark on Protest

☐ The additional search fees were accompanied by applicant's protest.

☐ No protest accompanied the payment of additional search fees.