Biocompatibility between tissue-engineering diamond-like carbon film and human vascular endothelial cells*★

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Abstract

OBJECTIVE: To verify the biocompatibility between diamond-like carbon (DLC) film and human vascular endothelial cells and to provide evidences for construction of artificial mechanical valve prosthesis.

METHODS: Nanophase DLC film was deposited using pulse laser deposition, while vascular endothelial cells derived from human umbilical vein was cultured with nanophase DLC film *in vitro*. Cell growth and adhesion were observed under inverted microscope, and cell proliferation was measured with MTT method. In addition, levels of nitric oxide (NO) and prostacyclin (PGI_2) were measured in the DLC group and blank control group in order to evaluate their activities.

RESULTS: Adhesion, proliferation, and growth of vascular endothelial cells derived from human umbilical vein were great on the surface of nanophase DLC film. There were no significant differences in the levels of NO and PGI_2 between DLC group and blank control group ($P > 0.05$), showing that nanophase DLC film had no effect on activity of vascular endothelial cells derived from human umbilical vein.

CONCLUSION: Nanophase DLC film has a good biocompatibility, and it can become an ideal material of tissue-engineering artificial mechanical valve prosthesis.

INTRODUCTION

Diamond-like carbon (DLC) is a hydrocarbon that possesses a lot of carbonic key like construction of diamond. DLC is deemed to an ideal material to manufacture mechanical heart valve[1-9]. Asinberg and Chabot firstly exploded a bunch of carbon on substantial cathode to attain it under vacuum condition of 13.3×10^{-5} Pa in 1971. Since 1990s, researches have become more and more widely. DLC can be sedimented on almost all materials below 250 ℃, whose configurable performance is adhere to bumping level of high-speed ionic. The research of biocompatibility of DLC membrane is flourishing recently, which discovers a very much valuable outcome that platelet was absorbed very hardly on it. Because biologic behavior of biomaterials implanted has something with outcome that superficial membrane and body interaction, the superficial characteristics consequentially affect its biocompatibility, in particular anticoagulant property. To be mentioned, it has been proved that highly abrasion resistance and well biocompatibility degrade enormously wear and tear, accordingly improve immensely the function of implanted materials[4-14].

Now, DLC membranes can get across many means to preparation, for example ion beam deposition (IBD), ion beam enhancement deposition (IBED), radio frequency sputtering (RFS), magnetron sputtering (MS), vacuum cathode arc welding sediment, direct current-glow discharge (DC-PCVD), radio frequency-glow discharge (RF-PCVD), pulse laser deposition (PLD), *etc.* Different mean differences are prodigious. But from application angle to see, the most important things are temperature of sediment, area of sediment, and velocity[1-11]. Therefore, PLD is the better means of sediment, which can deposit many complicate ingredient under less temperature of basic-board, especially fit membrane with severe construction, and intensive materials are easily prepared, and ingredients are expediently adjusted, and growth parameter are independently control, and chemical computation is exactly master. When DLC membrane lives up to nm ranged by PLD, the characters will obviously change and be entirely different of massive substance.

This biomaterial secures the efficacy and safety of clinical application. So the cell compatibility is the capital request to tissue engineering. The nanophase with DLC film compound materials are cultivated in vitro and we investigate cell compatibility, by nanophase with DLC film compound materials.

MATERIALS AND METHODS

Materials

The nanophase with DLC film compound materials were provided by Hefei University and Chinese Technology University, materials which were prepared to the size with 10 mm × 5 mm × 1 mm, 10 mm × 5 mm × 5 mm and 5 mm × 5 mm × 1 mm. The belly stalk was provided from health parturient within six hours after cesarean section. This study was approved by the *Administrative Requlations on Medical Institution*[15].

Reagent	Source
DMEM culture medium, newborn calf serum	Gibco Company, USA
Collagenase type I, trypsin, vascular endothelial cell growth factor, M199 culture medium, rat-anti-human α-actin antibody	Sigma Company, USA
Fetal bovine serum	Sijiqing Biology Co., Ltd., Hangzhou, China
Ⅷ factor-correlated antigen antibody	Zymed Company, USA

Methods

Isolation, culture and identification of human umbilical vein endothelial cells

Umbilical cord of health parturien was taken after

cesarean within 6 hours. They were cultured according to Jaffemethod after improving the method. In sterile condition [4], a 20-25 cm length belly band was superscripted before cells digestion. The bloodstain was removed on tunica adventitia of umbilical cores, finding the entrance to umbilical vein, inserted silica gel channel and ligatured tightness with line. By the same manner way, a silica gel channel was ligatured in the other end of umbilical vein, and then injected into lumen of vein by washing white block with basic PBS repeatedly with syringe until belly band became pallor and PBS without bloodstain. 0.1% collagenase type I was injected into lumen of vein to fill the vein. The two tip cannula was clamped with hemostat and incubated at 37 ℃ with 5% CO_2 for 18-20 minutes (tumbling one time during intermedium) after checking without enzyme leakage. The liquid was collected into centrifuge tube and it was centrifuged (800-1 000 ×*g*, 10 minutes). The supernatants were scrubbed, and then capture cells were deposited and inoculated 1×10^6/mL into culture bottles after centrifugation and washing. And then the M199 medium was supplemented with penicillin sodium 200 U/mL, streptomycin 0.2 μg/mL, and 20% newborn bovine serum. They were incubated at 37 ℃ with 5% CO_2, went down to posterity after 5-7 days and preached one generation after 3-5 days. Cells of 2-4 generation were taken to exert experiment and cells of each generation kept one exponent by immunohistochemistry assay to detect the expression of Ⅷ factors.

Cell cultivation

2-4 generation of the human umbilical vein vascular endothelial cells which grow well and stable were taken in this study. The cells were digested by pancreatic enzyme and counted of 6×10^6/L density. The cells suspensions were infused into nanophase membrane with DLC film compound material, and then they were stetted for 2-3 hours and immersed by DMEM culture solution with 10% fetal bovine serum. Later they were put into cultivation device to cultivate.

Observation under the inverted microscope

The nanophase membrane with DLC film compound materials were coated by artificial heart mechanical valve prosthesis with the size of 10 mm × 5 mm × 1 mm. The pre-wetting materials were put into the cultivation discus of six holes. The density of 2-4 generation of the human umbilical vein vascular endothelial cells was seeded on the pre-wetting materials. Then cells growth circumstance on the materials was observed under inverted microscope, after adding 5 mL culture solution after one hour incubation.

Observation under the scanning electron microscope

The nanophase membrane with DLC film compound materials were coated by artificial heart mechanical valve prosthesis with the size of 10 mm × 5 mm × 1 mm. The cells were cultured on the pre-wetting materials and the materials were taken after culturing 3 or 7generation. The cells were fixed by 2% glutaraldehyde and dried at critical point, later plated with gold. Then the coherence state on the materials was observed.

Observation of the confocal microscopy of light amplification by stimulated emission of radiation

Three nanophase membrane with DLC film compound materials coating by artificial heart mechanical valve prosthesis with the size of 10 mm × 5 mm × 1 mm were dipped into the solution of 1% Rhodamine for five minutes, steeping in distilled water for 30 minutes. They were torrefied and irradiated with ^{60}Co for degermation. The human umbilical vein vascular endothelial cells were stained by 60 μm DAPI /per unit for 30 minutes and washed three times. They were cultured on the materials which were stained by Rhodamine, and then the coherence state was observed after one week on the materials under the confocal microscopy of light amplification by stimulated emission of radiation.

Determination of cell activity by MTT assay

A total of 42 pieces of materials with the size of 5 mm × 5 mm × 1 mm were taken into cultivation discus of 96 holes for pre-wetting. Each hole was put a piece of material for 42 holes and another 42 holes were blank group without material. The human umbilical vein vascular endothelial cells were cultured into the materials and blank holes for incubating about one hour. Every hole was added into 2 mL culture solution to continue to cultivate. Everyday six holes of each group were put 20 μL MTT (5 mg/mL) in it and ended cultivation after 4 hours. the supernatant fluid of the holes were thrown away, then each hole was added 150 μL DMSO and oscillated for ten minutes in order to dissolve the crystal thoroughly. The absorbability of each hole was measured by enzyme immunodetection equipment at the wave length of 540 nm and the growth curve of cells multiplication was described.

Secretion level of NO and PGI_2 of vascular endothelial cells

Eighteen pieces of materials with the size of 10 mm × 5 mm × 1 mm were taken into cultivation discus of six holes for pre-wetting. Each hole was put a piece of material and another 18 holes were blank groups without material. The human umbilical vein vascular endothelial cells were cultured into the materials and blank holes for incubating about one hour. Every hole was added into 5 mL culture solution to continue to cultivate. Collecting the medium of 2, 5, 8 and 11 days, they were assayed with the methods of NO kit and 6-keto-$PGF_{1\alpha}$ kit.

Levels of NO and PGI_2

Concentration measurement of nitrogen monoxidum (NO): As the description of NO kit, the levels of NO which secrete from endothelial cells of DLC material groups and blank groups were assayed for 2, 5, 8 or 11 days.

PGI_2 of radioimmunodetection of endotheliocyte secreting: Collecting the medium of DLC material groups and blank groups for 2, 5, 8 or 11 days, they were placed into -20 ℃ refrigerator to conserve after centrifugation. The specimens were collected and they were assayed the content of 6-keto-$PGF_{1\alpha}$ as the content of PGI_2.

Explantation experiment

The simple artificial mechanical prosthetic valve and nanophase membrane with DLC film compound materials were over-laid on mechanical valve prosthesis of artificial heart. Then inserting them into canine atrium dextrum, we found that the heart vascular endothelial cells cohered to surface of membrane after three weeks. The result showed that

perspective of this research was light.

Statistical analysis

SPSS 10.0 software was used in this study. Experimental data were expressed as Mean ± SD, and measurement data were compared with analysis of variance. Level of significance was 0.05.

RESULTS

Observation of umbilical vein vascular endothelial cells of primary generation

The umbilical vein vascular endothelial cells of origin generation were spherical under invert microscope. They showed that one or a small mass and their nucleus took on round or ellipse, having one or several nucleolus (Figure 1).

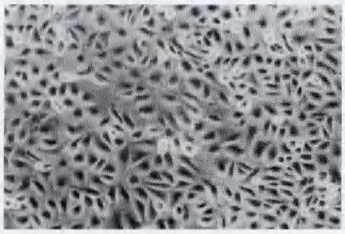


Figure 1 Human umbilical vein vascular endothelial cells (×100)

Cell growth

The materials were light tight, so superficial cells of the materials were difficult to observe under the inverted microscope (Figure 2).

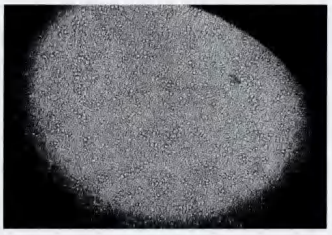
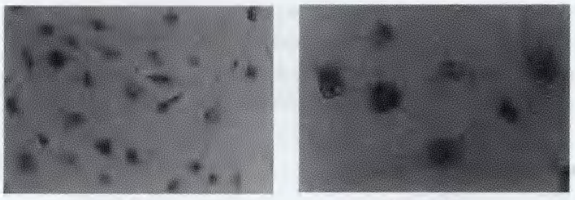


Figure 2 Growth of human umbilical vein vascular endothelial cells on the surface of nanophase membrane with diamond-like carbon materials (Inverted microscope, ×200)

Vascular endothelial cells were deposited, it was thus clear that there were brown granules in plasma of positive cells appeared by PAP method (Figure 3).

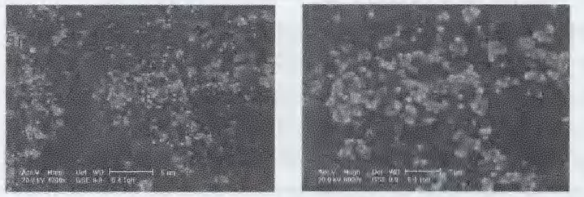


a: ×200 b: ×400

Figure 3 Artificial heart instrument of valve sediment diamond-like carbon film have human umbilical vein vascular endothelial cells, the yellow and brown granules in cell plasma were confirmed by von Willebrand factor expression (PAP staining)

Cell adhesion under the scanning electron microscope

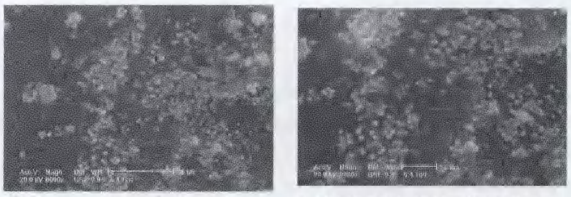
The cells on the surface of DLC material and in ventage cohered after combined cultivate for five days. They already extended to fusiform or polygon and exserted protruse pseudopod to adhere materials. Copious microvilli were sawn on the surface of the cells, showing the cells were in good condition (Figure 4).



a: Cells grow extensively and stretch out multiple pseudopods b: Vascular endothelial cells are adherent to the surface of material

Figure 4 Human umbilical vein vascular endothelial cells on artificial heart instrument of valve sediment diamond-like carbon film material on the fifth day (×2 000)

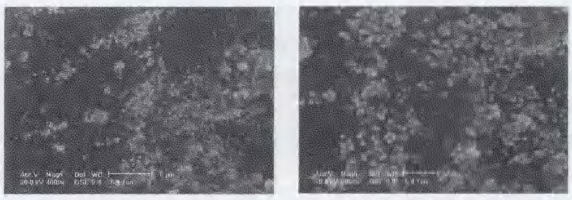
Cellular quantum increased after combined cultivate for eight days and crystallizations of granulo-calcium salts deposited on the surface of the cells (Figure 5).



a: Cells grow extensively and stretch out multiple pseudopods b: Vascular endothelial cells are adherent to the surface of material

Figure 5 Human umbilical vein vascular endothelial cells on artificial heart instrument of valve sediment diamond-like carbon film material on the eighth day (×2 000)

They were connected and mixed each other, and appearance of the cells was not obviously different (Figure 6).



a: Cells proliferate increasingly and connect to each other b: Cells proliferate increasingly and connect to each other

Figure 6 Human umbilical vein vascular endothelial cells on artificial heart instrument of valve sediment diamond-like carbon film material on the eleventh day (×2 000)

Cell proliferation

The situation of cell multiplication on the materials might be sawn growth curve drawing by the absorbance of the cellular activity series. The cytoactive of DLC materials group and blank group increased gradually after the calculation of cells. Two groups remained normal speed of division growth and were unknown significance difference ($P > 0.05$, Figure 7).

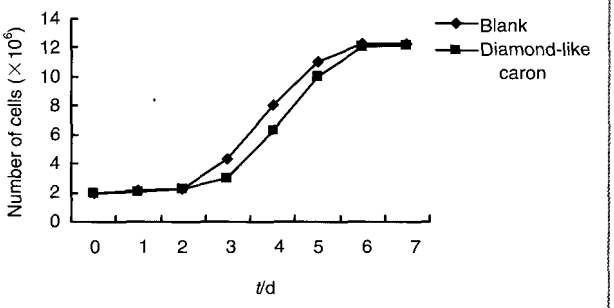


Figure 7 Growth curve of cell proliferation

Level of NO and PGI_2

NO and PGI_2 secreted from vascular endothelial cells of human umbilical vein were not significantly different between the DLC materials group and blank group ($P > 0.05$), showing that DLC material was not unfavorable effect on vascular endothelial cells of human umbilical vein and the difference in their activity (Table 1).

Table 1 Level of nitric oxide (NO) and prostacyclin (PGI_2) ($\bar{x}\pm s$)

Group	NO (μ mol/L)			
	2 d	5 d	8 d	11 d
Diamond-like carbon	80.98±5.98	81.98±6.98	82.98±5.98	84.98±4.98
Blank control	81.92±6.92	81.92±6.84	83.45±5.94	84.52±4.83
Group	PGI_2 (μ g/L)			
	2 d	5 d	8 d	11 d
Diamond-like carbon	16.41±0.53	25.73±0.59	21.43±0.39	17.59±1.29
Blank control	16.21±0.56	25.53±0.57	21.67±0.59	17.58±1.32

DISCUSSION

The biocompatibility of material is physics of ectochemistry in nature and contains blood compatibility and histocompatibility. The blood compatibility especially anticoagulant property of material which could contiguity with blood straight is important. Biomaterial get in touch with bodies in short term or long-term, even lifetime, so the evaluation of biomaterial compatibility is most significance. Now the evaluation of biomaterial compatibility main localize into two layers[7-11]: one is the methods *in vivo*, the materials are inserted into animal bodies, then the growth conditions of tissue are surveyed after a period of time. The other is the methods *in vitro*, cellular sticking, stretching, growth and biological character of simple *in vitro* model outwardly are investigated. The chief evaluation means of biomaterial compatibility still is implanted experiment in animal *in vivo*, but simple implanted experiments *in vivo* do not satisfy with requirements of material development increasingly with continual deeply investigation and their limitations manifest day by day. First of all, cycle time of experiments *in vivo* is too long, process tedious, time consuming. They do not satisfy with high performance and fast screening demands of generous material during the tentative design. Secondly, factors *in vivo* are multiplicity and uncontrolled. Specificity information of some materials factors does not take accurate, so precisely guidance opinions are impossible provided to further optimum design. Thirdly, time points are often selected to review implant button after material being implanted *in vivo*. If they are improper, magnanimous experiment information may be neglected. Experiments *in vitro* manipulate relative simple and controllability is powerful, so they approach single factor of material on organism effects. The method reproducibility is better and has no effects on vulnerarious of operations. There are a world of potence on overview reactions of materials and cells after spurt touch. On account of above reasons, evaluation system *in vitro* develops quickly in short time and it becomes a chief method to investigate biomaterial compatibility.

The shade selection experiment with tetrazolium salts (MTT) is proposed by Mosroann in 1983 and it first is applied in immunology domain. Some scholars recent years apply this methods in biocompatibility evaluation to detect cellular toxic action. Its principal utilize fumaric reductase of living cells chondrosome to deoxidize ectogenic tetrazolium salts into indissolvable amethyst crystals and the crystals deposite in intra-cellular, dead cells not having this function. Dimethyl sulfoxide could resolve intrastitial crystal and light absorbabilities are determined indirect to reflect viable count. The sensitivity of this method is high and operation is simple and reproducibility is well. Experimental results show that corpuscular proliferation of inoculated group have no harmful effects after recombinating a week. This elucidate DLC material has atoxic action on human cells.

Vascular endothelial cells are not only barrier of endangium, but also high athletic metabolism and endocrine cells. They could adjust vascular permeability and secrete multiple vasoactive substance, such as NO and PGI_2, to adjust vasoconstriction and blood clotting fiber melt down system equation. Meanwhile they partake in organism metabolism and surface of vascular endothelial cells usually do not thrombose, bec-ause normal endothelial cells have many antiembolic functions for relating Antithrombin-Heparan (AT-III HEP) of endothelial cells. Endothelial cells may synthesis and excrete many vasoactive substance, for example PGI2 and EDPF to expand vascular material. Both of them could inhibit platelet aggregation and depolymerize accumulative blood disk. PGI_2 also could rivalry thromboxan A2 and form. Matter to inhibit platelet adhesion and collection, endothelial cells form 6-ketone PGE1 and 13-hydroxyl-octadecenic acid except PGI_2, EDRF to inhibit platelet adhesion and collection, also destroy active mass which promote platelet aggregation, for example 5-HT. Membrane contains ADP enzyme (which would hydrolysis ADP and produce AMP, the latter inhibit platelet aggregation). There are antithrombase materiae: ① mucopolysaccharide: heparan sulfate (binding AT-III with inactivating factor IXa, Xa, XIIa) and slight chondroitin sulfate (binding heparin cofactor-II to speed up and counteract plasmase); ② AT-III; ③ TM: TM is a high affinity plasmase receptor on the surface of EC. It is a cofactor which quickens the speed of plasmase activating PC into APC, and the latter inactivate factor Va, VIIIa and cohere plasmase to inhibit activation of blood coagulating protein and plasmozyme activation of thrombase. It contains aminogalactose which accelerates AT-III to inactivate

thrombase. So the normal endothelial cells main display on the anticoagulation and preventing thromb.

The application of laser scanning microscope could direct observe morphous of living cells of vascular endothelial cells of material. It remains maxlmum extent; interrelationship of material and cells. The object within the scope of scanning according to definite step pathway stepwise scans according to definite step pathway gradually scanning. Scanning patterns are built up through computer eventually, so three-dimensional reconstruction would implement. We not only observe cells of super material, but also internal cellular distribution material ecderon and sticking and accretion of vascular endothelial cells on super HA material and secretary level of vasoactive substance, for example NO and PGI_2. Secretary level of NO and PGI_2 of endothelial cells are compared which are collected the HA material group and blank group on 2, 5, 8, and 11 days. They show material is avirulent for cell and has favorable biocompatibility. Corpuscular functions of DLC material are well and this provides bases on constructing mechanical valve prosthesis materials with tissue engineering methods.

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组织工程化类金刚石膜复合材料与人血管内皮细胞的相容性研究*★

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摘要

目的：验证纳米相类金刚石薄膜复合材料与人血管内皮细胞相容性，为组织工程化机械瓣膜材料的构建提供依据。

方法：利用脉冲激光沉积法在人工心脏机械瓣膜上沉积纳米相类金刚石薄膜，将人脐静脉血管内皮细胞与纳米相类金刚石薄膜复合材料体外复合培养。倒置显微镜及扫描电镜观察细胞在材料表面的生长、附着情况；MTT 法检测细胞在材料上增殖情况；同时分别测定人脐静脉血管内皮细胞在类金刚石薄膜材料和空白对照组中一氧化氮及前列环素分泌水平，以评价其活性。

结果：人脐静脉血管内皮细胞能在纳米相类金刚石薄膜复合材料上良好地黏附、增殖、生长。人脐静脉血管内皮细胞分泌一氧化氮和前列环素水平在类金刚石薄膜材料和空白对照组没有显著性差异($P > 0.05$)，说明纳米相类金刚石薄膜材料对人脐静脉血管内皮细胞的活性没有影响。

结论：纳米相类金刚石薄膜复合材料具有良好的细胞相容性，有可能作为组织工程化机械瓣膜材料。

关键词：脉冲激光沉积；类金刚石膜；组织工程；人血管内皮细胞；细胞培养；细胞相容性

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