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Usefulness of the Behavior of Fibroblast Attachment to Coils in Thermoreversible Gelation Polymer for Aneurysmal Coil Treatment

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Abstract

Background: Post embolization aneurysm reccurence is still a drawback in endovascular coiling for cerebral aneuryms. That stimulated reaearch into new promising embolic materials, as the thermoreversible Gelation Polymer (TGP) when used as cell culture medium with its temperature related properties. We evaluated proliferative properties of fibroblast cell cultures in association with metalic coils.

Methods: Intial assessment of cell viability was done for 3T3 (mouse fibroblast) and A172 (glioma cell) lines for 6 and 16 days in TGP solid phase and media. After that TGP was liquified and cell attachment confirmed. Next, fibroblasts were cultivated under the same conditions adding Guglielmi detachable coils for 24 h and 3 days with TGP, evaluating cell attachment and proliferation on coils. Last, the time course of cell proliferation was evaluated, comparing cell numbers on equal coil surface areas for 1,3,5,7,10,14 and 21 days in TGP. Electron microscopy was used for cell assessment on the coils.

Results: Fibroblasts survive and show satisfactory viability in TGP. In liquified TGP after 6 days and 16days, both cell types temporally extedned processes and attached on flask`s bottom. Next after 24 h and 3days fibroblast cultivation with coils in TGP, cells exteded processes and attached to coils, indicating some proliferation and clustering. Last, the day 1 attached cell numbers on coils cultivated in TGP guradually decreased until day 10 and later increased again to just below day 1 level. However, both fibroblast and glioma cells in TGP did not extend processes, move or proliferate, and had spherical shape while in solid TGP.

Conclusion: Fibroblasts attachment and long survival on coils in TGP may facilitate aneurysm treatment to achieve better volume embolization rate and improve intra-aneurysmal thrombus organization.

Keywords Fibloblast; Thermoreversible gelation polymer; Coil; Aneurysm

Introduction

Subarachnoid haemorrhage (SAH) due to rupture of cerebral aneurysm is a significant cause of morbidity and mortality throughout the world [1]. Incidence of SAH is between 5 and 10 per 100 000 in a number of studies in 1990s [2]. Mortality rates vary widely and the median mortality rate in epidemiological studies from the United States has been 32% versus 43% to 44% in Europe and 27% in Japan [3]. Rerupture of aneursms also leads to very high mortality and poor prognosis for functional recovery in survivors and its prevention is extremely important.

Aneurysms are treated with microsurgical and/or endovascular methods. Endovascular therapy becomes increasingly popular alternative to surgical clipping because of being less invasive and the accumulation of results indicating similar or better outcomes [4,5]. However it has been less effective in wide-neck or large/giant aneurysms. Main difficulties in aneurysm embolisation are the achievement of complete obliteration of the sac and neck orifice, as well as occurrence of coil compaction and recanalisation of the lesion later. The mechanisms of aneurysm recanalization remain unclear. However the contributing factors to prevent recanalisation are high volume embolization rate, promotion of thrombus organization in the aneurysm and endothelialial coverige of the occluded aneurysm orifice [6].Volume embolization ratio greater than 25% was necessary to achieve stability in large and wide-neck aneurysms treated by detachable platium coil embolization [7] and currently it is a routine goal during treatment. As an additional factor, the volume of the aneurysm and induced thrombus respectively have different times of maturation, being longer the bigger the lesion. In some wide-neck or large/giant aneurysms, the thurombus induced by coil placement remains poorly organized after 3 weeks from treatment. In small aneurysms within the first week post-treatment few fibloblasts and non-organized thrombus were present, but during the second week fibroblastic ingrowth was more prominent within the advancing from the periphery granulation [8]. Fibloblasts accelarated early histlogical profliferation/maturation compared to controls [9,10].At the completion of the process, tissues obliterating the aneurysm neck orifice were usually covered by neoendothelial membrane [11], replacing the surface of the initial fresh thrombus [12]. Thus, stable aneurysmal thrombosis is demonstrated by continuous endothelial lining between already occluded neck orifice surface and patent artery lumen [11]. The formation of mature thrombus within the aneurysm

apparently contributes to endothelialization [13]. In summary, these reports suggest that in addition to high volume embolization, proliferation of fibroblasts and endothelial layer for the treated aneurysm and its orifice are important factors for avoidance of aneurysm recurrence.

In an attempt to reduce recurrences, new non adhesive liquid embolic agents have been evaluated for aneurysm treatment. The thermoreversible gelation polymer (TGP) is an aqueous solution that remains liquid at temperatures less than the sol–gel transition temperature (TT) and becomes a gel at temperatures higher than the TT. TGP was originally developed as a cell culture medium [14,15] and can be used as a drug delivery vehicle for chemotherapy [16,17] or active substance carrier for basic fibroblast growth factor together with live fibroblasts [18]. Our group has already reported TGP as a new thrmoreversible liquid embolic agent for treatment of experimental aneurysms [18-21]. Intending to enhance the process of embolic material stabilization after endovascular aneurysm treatment, we evaluated the behavior of fibroblasts in TGP and in relation to bare platinum coils, simulating the eventual application in a treatment scenario.

Materials and Methods

Preparation of thermoreversible gelation polymer (TGP), cell culture method and coils

TGP, Mebiol Gel, MB-10; was provided by Mebiol Inc., Tokyo, Japan, as freeze-dried powder. Allocated TGP for 25ml culture flask was mixed with 10ml of Dulbecco's modified Eagle's medium (DMEM, Invitrogen, Carlsbad, CA, USA) containing 10% fetal bovine serum (Hyclone, Logan, UT, USA) and antibiotics (100 μg/ml penicillin and 100 μg/ml streptomycin (Invitrogen)) and left for 3 days at 4°C for elimination of air bubbles.

Mouse fibroblast cell line, 3T3, American Type Culture Collection Rockvulle, MD (ATCC) and human glioma cell line, A172 (ATCC), were harvested after dispersing by trypsin. Malignant glioma A172 was also tested because the cell line is also adhering and not tumorigenic in immunosuppressed mice, however forms colonies in semisolid medium [22]. The centrifuged pellets were suspended in 0.5 ml of TGP on ice and cells of 5×10^4 for cell survival or 5×10^5 for cell proliferation and survival duration were plated on 35mm dishes and left in a 37°C in incubator for few minutes for gelling. Three ml of DMEM were added to cover the top of TGP and plates were further incubated and used for the study.

Six mm×20cm Guglielmi detachable coils (GDC)-10, bare coil, were cut in approximately 1cm segments. They were sterilized by irradiation (Figure1A).

Study for cell survival, cell proliferation on coils, and cell survival duration

Cell survival

Both 3T3 and A172 cells were cultivated in TGP for 6 days or 16 days. TGP was changed into fluid phase by cold DMEM. Cells were re-seeded for 2 days and observed by a charge-coupled device image sensor (VB7010, Keyence Japan, Osaka, Japan).

Cell proliferation on coils

3T3 cells with coils were cultivated for 24 h and 3 days in TGP (three coils for each culture) (Figure 1A, 1B). TGP was solved after the cultivation and coils were immediately pulled out and submersed in 1.2% glutaraldehyde in 0.1 M phosphate buffer (pH 7.3) at 4°C for fixation of cells as pre-treatment for electorn microscopy.

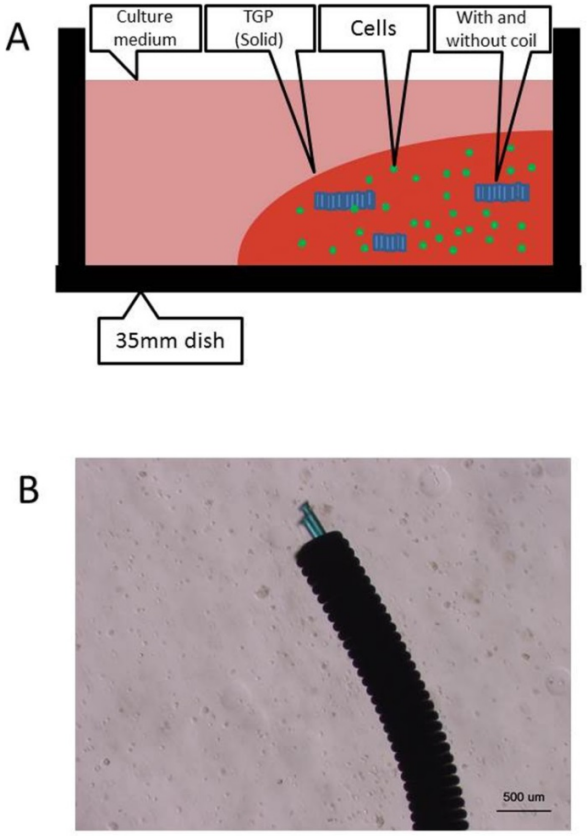

Figure 1: 3T3 cells and coil in TGP (A) schemas, (B) on day 1 culture. Bar = 500 μm.

Temporal pattern of cell survival

Cells with coils were cultivated for 1, 3, 5, 7, 10, 14 and 21 days in TGP (six coils for each time period except day 21 when three coils were used). An additional culture of 1day without TGP was performed as a control, using 3 coils. After the cultivation, coils were submersed in 1.2% glutaraldehyde for electron microscopy. Overall architecture of each coil was demonstrated by scanning electron microscopy. Cells extending processes to coil surface were counted for coil length of 25 turns of the covering wire. Coils with too much gel attached were excluded.

Electorn microscopy

After specimens were fixed by treatment with glutaraldehyde for few days, the coils were dehydrated with a graded series of ethanol from 50% through 70, 80, 90 and 100%. Following further treatment with 100% iso-amylacetate, the coils were prepared in a critical point dryer

(Hitachi High-Technologies Corporation, Tokyo, Japan) and sprayed with Au-Pd. Coils were examined at 15 kV under a JSM-5800LV scanning electron microscope (JEOL Ltd., Tokyo, Japan).

Results

Survival and behavior of fibroblasts in TGP

Both 3T3 and A172 cell populations survived in both 6 day and 16 day cultures. 3T3 cells in TGP did not extend processes, move or proliferate for 5 days (Figure 2A). In few observations at higher magnifications their shape was spherical (Figure 2B). Cells in TGP after being cultivated for 16 days were re-seeded for 2 days under the normal conditions of cultivation after TGP was solved.

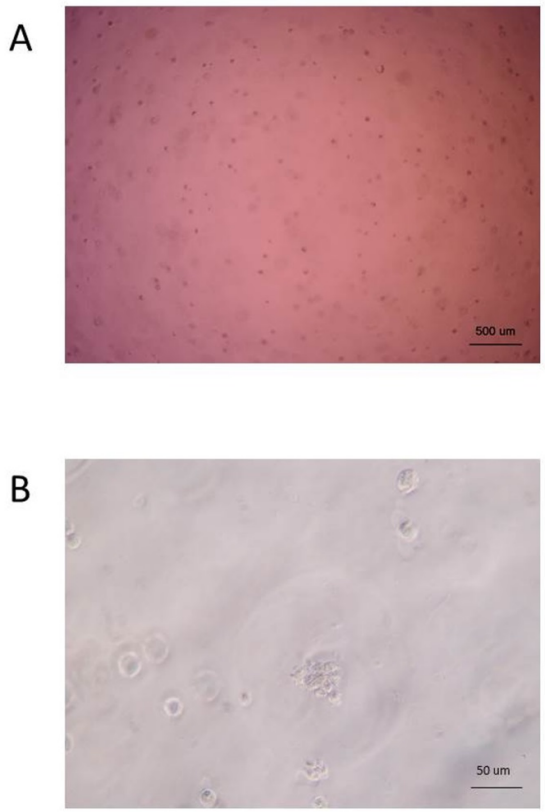

Figure 2: 3T3 cells on day 5 in TGP (A) cells were small round shaped and seemed not to move freely. Bar = 500 μm, (B) cells made spheroids. Bar = 50 μm.

Large number of dying cells were observed but cells with temporarily extedned processes and attached to the bottom of the flask could be also observed (Figure 3A). We obverved the same phenomenon in the A172 culture ,which did not proliferate without scaffolds as the 3T3 did, and had stronger proliferative properties (Figure 3B).

Attachment and proliferation on coils in TGP

At 24 h cultivation, a small number of cells with coils in TGP exteded processes and attached to coils (Figure 4A). More, compared with 24 h, cells cultivated for 3 days in TGP seemed to proliferate and made partially small clusters. Cells did not overlap but were located very near each to other (Figure 4B).

Temporal pattern of cell survival

The relation of survival time and the number of surviving cells on coils in TGP is shown in Figure 5. The number of cells on coils cultivated for 1 day in TGP was approximately 59% of the number when cultivated without TGP as a control. After day 1 the number of cells on coils cultivated in TGP gradually decreased until day 10, later to increase again by day 21 to below day 1 levels, but extending processes.

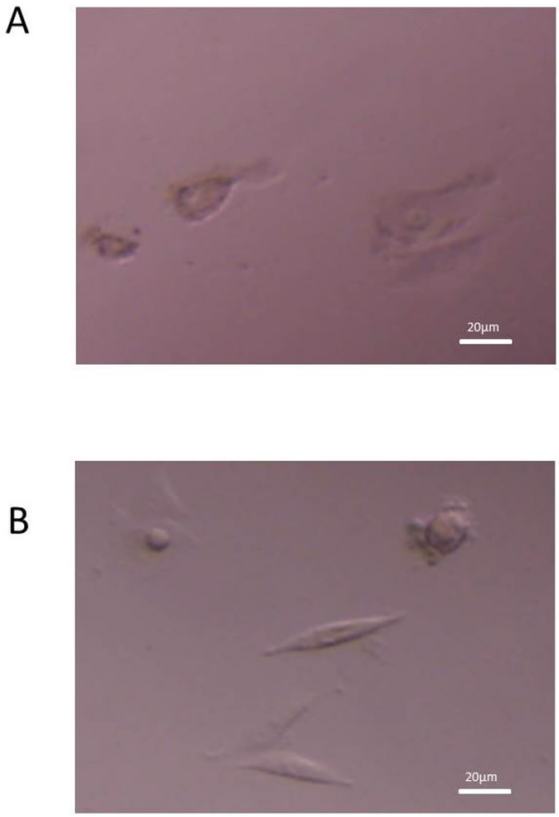

Figure 3: Survival of cells in TGP. (A)(B) Cells were observed by phase contrast microscopy two day aftre cultures for 16 days. Bar = 20 μm (A) 3T3 cells. (B) A172 cells.

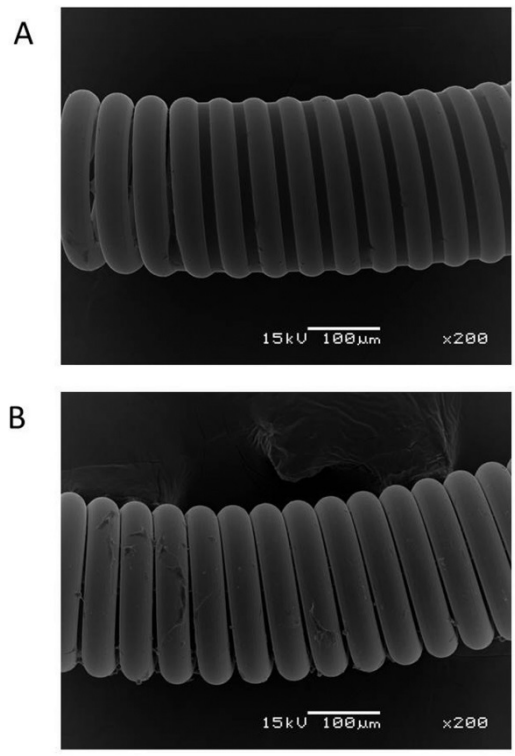

Figure 4: Cells proliferation in TGP. 3T3 cells growing (A) for 24 h and (B) for 3 days, (B) cells seemed to increase and made partially small clusters.

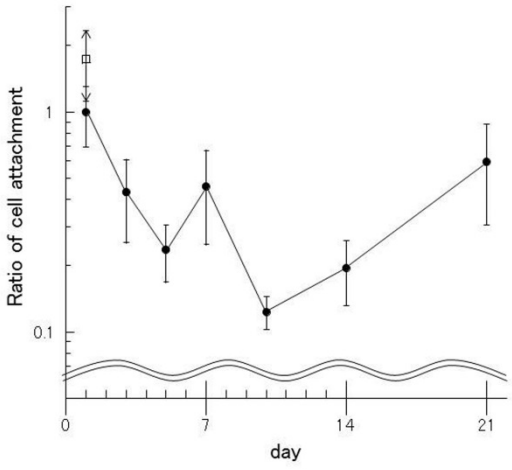

Figure 5: Duration of attachment and number of proliferation. The number of cells per 25 turns of coil counted on days 1, 3, 5, 7, 10, 14 and 21 in TGP (•)and on day 1 in normal conditions (□), compared with on day1 in TGP. The number of cells cultivated for 1 day in TGP compared with normal condition was about 59% of the cells. Bars=SE.

Discussion

Our study showed that 3T3 cells on coils, even in TGP, proliferated with extending processes and making clusters. Moreover, in TGP nearly half of 3T3 cells seen in normal cultivation attached to coils 1 day after cultivation. Initially the rate of 3T3 attachment decreased, but from day 10 it increased again until day 21. These results are potentially applicable if we compare this time course with the time course of aneurysm thrombus organization after coil treatment.

Packing of more of 25% to obtain high volume embolization rate prevents unstability of occluded aneurysms such as recanalization [7].However, in aneurysms with a volume of more than 600 mm3, such high VER could not always be obtained [23]. TGP used in our experiment is characterized by its temperature-dependent dynamic viscoelastic properties and lack of pre-determined shape because of its fluid phase [24]. We demonstarated already that TGP at low temperatures could be injected through a catheter for safe embolization of aneurysms [19-21]. TGP injected in aneurysms embolized by coils as an additional step may be useful for increasing of packing density and preventing recanalization.

The promotion of wound healing and thrombus organization inside aneurysms has been studied using protein coated coils [25] and bioabsorbable polymer coils [26]. In small aneurysms, a large number of fibroblasts and very small amount of old clot in the central aneurysm portions were detected less than 14 days after embolization. On the other hand, in large aneurysms, varying amount of old clot in the central aneurysm portions were still detected about 21days after embolization. Organization in large lesions took longer time and seemed to create difficulties for fibroblasts to ingrow [8]. Fibroblasts delivered in aneurysms could promote the organization in aneurismal thrombus [9, 10], even if they are in TGP environment [18]. TGP with fibroblasts injected into an aneurysm embolized by coils may also be useful for the promotion of organization.

In an in vivo model fibroblast survival has been explored [18], however the length of survival and behavior on coils in TGP has not been investigated. Our data give just the in vitro estimation of this process.

Endothelialization overlying the occluded aneurysm orifice needs the organization of the intraaneurysm thrombus [13]. Endothelial cells migrated using the extracellular matrix, such as collagen produced by fibroblasts as a scaffold [27]. In an "in-vitro" experiment, endothelial cells from the edges of damaged arterial endothelium in thrombosis models restore the endothelium by elongation, migration and proliferation [28]. In vivo however, endothelial overgrowth from the aneurysm orifice margins progressively covered the already fibrotically organised parts of the cloth inspite the presence of fresh thrombus in some parts of the occluded aneurysm [12]. In endothelialization a fibrin membrane across the orifice of aneurysm appeared 36 hours after embolization [29] and afterward endothelial cells seemed to elongate on that membrane [30]. This endothelialization is the final stage of aneurysm exclusion from the parent vessel circulation [11]. Endothelialization of the aneurysm usually occurred by 2 weeks after embolizations [31]. Our experiments showed fibroblasts in TGP attacheded to coils as a scaffold and proliferated after more than 14 days. The living fibroblast requires extracellular matrix as an anchorage and is activated there for proliferation. Fibroblasts induce endothelial cells through the synthesis and maintenance of the extracellular matrix [32] and may promote endothelailization.

TGP can have various applications such as a drug delivery vehicle for chemotherapy [16, 17] or a non-toxic cell culture medium [14, 15]. As a drug delivery system TGP can be used to induce endothalial cells with endothelial growth factor such as VEGF [33], deliver adhesion molecules such as integrin [34] or fibronectin [35] to mediate endothelial cell adhesion and inflammation inducing substances that might be useful for thrombus organization. The development of TGP can become an additional resource to prevent recanalization of aneurysms and embolize more effectively using several substances activating endothelial cells.

Some limitaions do exist before this result is applied to the treatment of cerebral aneurysums. One of them is the preparation of fibroblasts contained in TGP to be injected in the human. Cultured fibroblasts as those used in our study cannot be prepared shortly after an aneurysm rupture hospital admission, what may limit their application only in unruptured aneurysms. In addition we have not yet ensured complete safety of all components used for cultivation. We should use autologous fibroblasts harvested from the patients's subcutis, expecting less immunoreactivity from an easy to obtain specimen. Aneurysm embolization reflects only one application of the currently broadly used autologous fibroblasts in wound healing and tissue repair. As tissue organization in evolution is very important factor in effectiveness of aneurysm treatment, as a next step we should perform TGP embolization containing autogous fibroblasts and coils in vivo, adjusting the optimal fibroblast density in TGP and assess the aneurysm outcome angiographically and histologically.

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