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유기질과 무기질 복합체를 이용한 체내흡수형 인공골재료에 관한 연구
-세포배양에 의한 생체적합성 평가-

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=Abstract=

Biodegradable Inorganic-Organic Composite Artificial Bone Substitute
-In vitro biocompatibility evaluation by cell culture-

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A composite material was produced as an artificial bone substitute which is gradually degraded and replaced by the regenerated natural bones after implantation. To detect the effect of the material on the cell's activity, the composite specimens were placed in MEMs and incubated at 37℃ for one week. Human uterus cervical cancer origin HeLa 3 cells and mouse subcutaneous origin L929 cells were cultured in the specimen dissolved MEMs for 5 days to investigate cytotoxicity via cell growth rates. $Na_2{}^{51}CrO_4$ solution was added to the media, to label the HeLa S3 cells, and the released amount of ^{51}Cr was measured by a γ counter.

On the cell growth investigation, no significant cytotoxic phenomena were revealed in both HeLa S3 and L929 cell cultures. On the released ^{51}Cr from the incubated HeLa S3 cells, no significant cell degeneration was observed from the composite embedded MEMs.

Key words : Collagen, Carbonate apatite, L929, HeLa S3, Cytotoxicity

INTRODUCTION

An artificial bone substitute, which consists of a 3.82% carbonate contained apatite and a human umbilical cord origin type I atelocollagen, was designed as an inorganic-organic composite that would be gradually degraded and replaced by natural bone after implantation[1, 2]. *In vitro* mechanical property and biodegradability of the composite substitute were previously reported[3].

Cell culture has been a widely used and strongly recommended in vitro tool on investigating biocompatibility of the implantable materials[4, 5]. In this study, growth rate of the L929 fibroblasts and HeLa S3 cells in the composite dissolved minimum essential medium (MEM) were referred to cytotoxicity. The released amount of ^{51}Cr from the labelled HeLa S3 cells was also measured to investigate the cell degeneration rate[6].

MATERIALS AND METHODS

1. Preparation of the liquid specimen and cells

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Fetal calf serum (Hezleton Biologics Inc., Lenexa, KS, U. K.) was mixed with Eagle's Minimum Essential Medium (MEM: Sigma Co., St. Louis, MO, U. S. A.) to make 10% serum MEM. L-glutamin 2 mM/ml, penicillin 500 IU/ml and streptomycin 250 μg/ml were also added to the MEM.

2 mg of the composite specimen was placed in 10 ml of the MEM and incubated at 37℃ for one week, and the MEM was centrifugated at 200 × g. The MEM supernatant, that contains substances released from the dissolved composite, was used as the liquid specimen.

Human uterus cancer origin HeLa S3 cells and mouse subcutaneous origin L929 fibroblasts, supplied from ATCC, were cultured in Eagle's MEM for 3 days at 37℃ in a humidified atmosphere of 5% CO_2 and 95 % air. The cells were preincubated in the liquid specimen for 6 hours to let the cells be adapted to the media environment[7].

2. HeLa S3 and L929 cell growth in the liquid specimen

The preincubated HeLa S3 cells and L929 fibroblasts were evenly seeded on a 6 well plastic tissue culture dish (Corning Co., N. Y., U.S.A.), where 1 ml of the liquid specimen was placed in each wells. Numbers of HeLa S3 cells were controlled to 1×10^5 and 5×10^4, otherwise L929 cells were controlled to 5×10^4 for each wells, and incubated in a 5% CO_2 incubator at 37℃ for 5 days. Cell numbers were counted on a cell count plate after detaching cells by treatment with a calcium-magesium free salt solution containing 0.02% EDTA and 0.05% trypsin (CMF: Sigma Co., St. Louis, MO, U.S.A.). The composite dissolved liquid specimens were replaced everyday.

3. HeLa S3 cell labelling with ^{51}Cr

HeLa S3 cells were incubated in a 80 ml of MEM. After three days, 3 ml of CMF was added to the MEM, and incubated at 37℃ in a humidified atmosphere of 5% CO_2 and 95% air for 3 minutes to detach the cells from the culture dish. Another 3 ml of MEM was added to the dispersed solution, and centrifugated at 500×g for 5 minutes. Washing process was followed by removing supernatant and replacing with 1 ml of MEM. After washing twice, careful pipetting was followed to disperse the cells uniformly in the MEM. Cell numbers were controlled to 4×10^7 cells/80 ml MEM by cell counting method.

40 μCi of $Na_2{}^{51}CrO_4$ solution (radioactivity 200 Ci/g Cr: New England Nuclear Co., MA, U.S.A.) was added to the MEM, and the mixed solution was delivered to culture dish by 1×10^7 cells/80 ml MEM. Incubation at 37℃ in a humidified atmosphere of 5% CO_2 and 95% air was followed to let ^{51}Cr infiltrate into HeLa S3 cells.

4. ^{51}Cr counting

^{51}Cr labelled HeLa S3 cells were dispersed in and washed by MEM to remove excessive ^{51}Cr, that is not infiltrated into the cells. The cell numbers were controlled to 5×10^5/ml of MEM.

MEM was mixed to the composite dissolved liquid specimens, those were prepared on (1), to prepare 100, 75, 50, 25 and 10 v% liquid specimens. 10 mM of Hepes and 2.2 mg of $NaHCO_3$ were added to each 1 ml of liquid specimen.

1 ml of 5×10^5 ^{51}Cr labelled HeLa S3 cells sunpended MEM was mixed with 1 ml of the liquid specimens. As a negative control, 1 ml of MEM was mixed instead of the liquid specimens. These were incubated at 37℃ in a 100% humidity, 5% CO_2 condition for 4 hours.

1 ml of the incubated MEM was centrifugated at 500× g for 5 minutes, and 0.5 ml of supernatant was taken to assay the released ^{51}Cr quantity by a γ-counter for 10 minutes. The radioactivity of ^{51}Cr in the liquid specimen and the negative control was calculated according to the following formula.

$$\text{Released } {}^{51}\text{Cr ratio} = \frac{[\textit{supernatant 0.5 ml cpm}]}{[\textit{total } {}^{51}\textit{Cr control 0.5 ml cpm}]} \times 100$$

Each experimental group consists of 10 specimens.

RESULTS AND DISCUSSION

1. Cell growth in the composite dissolved MEM

In 1×10^5 HeLa S3 cells cultured group, there was no statistical significant differences on number of the grown cells between in the liquid specimen and control ($p > 0.05$) during 5 days observation (Fig. 1). But there were significant differences ($p < 0.05$) between them in each 5×10^4 HeLa S3 and L929 cells seeded groups. The cells in the

composite dissolved liquid specimens grew no less than in the controls (Fig. 2, 3). According to this results, it assumes that initial seeding numbers of cells less than 1×10^5/ml might be acceptable to investigate cytotoxicity by cell culture method.

During the preparation of the liquid specimens, not only apatite particles but also atelocollagen debris would be released form the incubated composite and dissolved in the MEM[3]. The results can be explained as that the dissociated apatite did not inhibit cell growth, while the dissolved type I atelocollagen promoted cell growth. Collagen is an extracellular matrix that contains cell adhesive Arginin - Glycine - Aspartic acid (RGD) protein sequence in molecule, and has been widely used as an additive to cell culture media[8].

For this experiment, a pepsine treated human umbilical cord origin type I atelocollagen has been used as a structural component of the composite to remove predictable immune response. Immune response of the collagen is related to the collagen molecular telopeptides, and proteolytic activity of pepsine dissociates the telopeptides from the molecular extremities[9]. Otherwise, both HeLa S3 and L292 cells are fibroblasts and type I collagen is abundant in the fibrotic tissues. For this reason, type I atelocollagen would provided a favorable environment for the cells growths in this experiment.

2. Released ^{51}Cr from the inhibited HeLa S3 cell activities

As the *in vitro* biocompatibility experiments, various kinds of cellular toxicity tests have been introduced. The

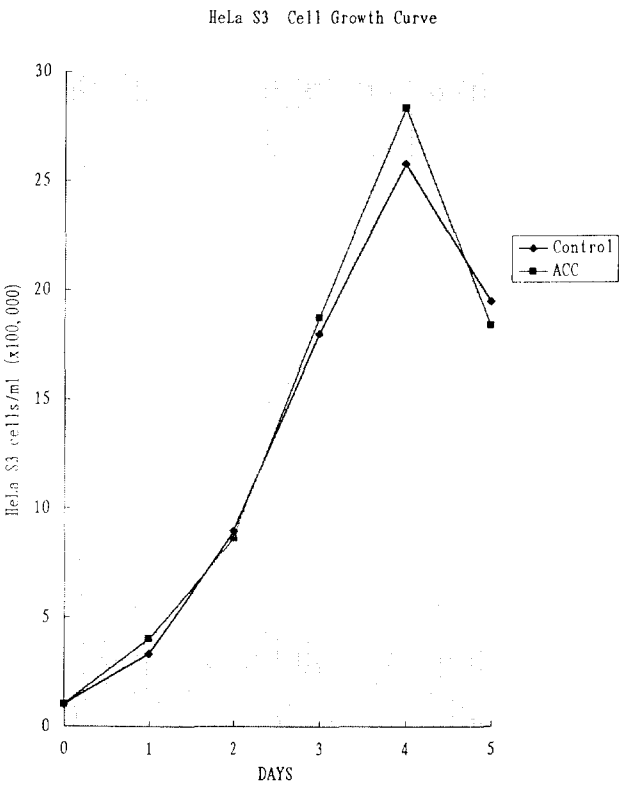

Fig. 1. HeLa S3 cell growth curve
initial seed : 1×10^5 cells/ml Liquid Specimen

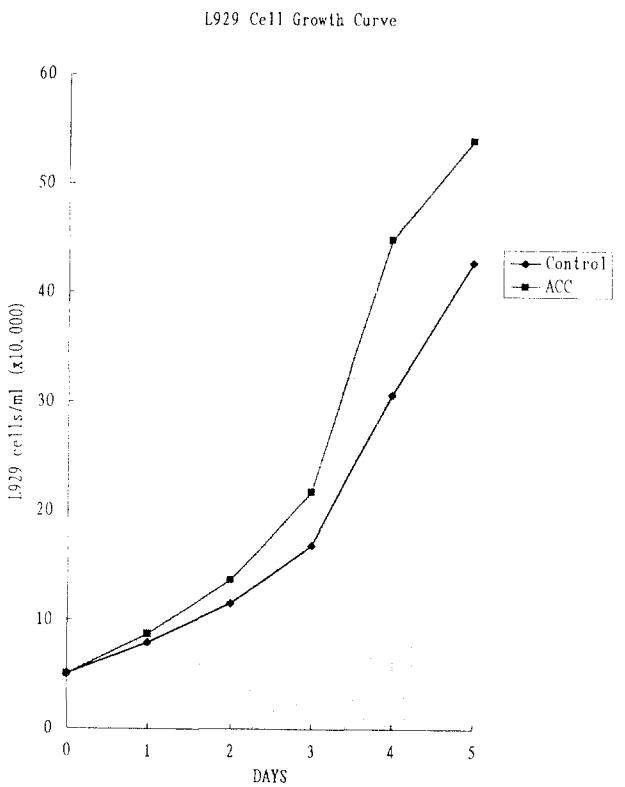

Fig. 2. HeLa S3 cell growth curve
initial seed : 5×10^4 cells/ml Liquid Specimen

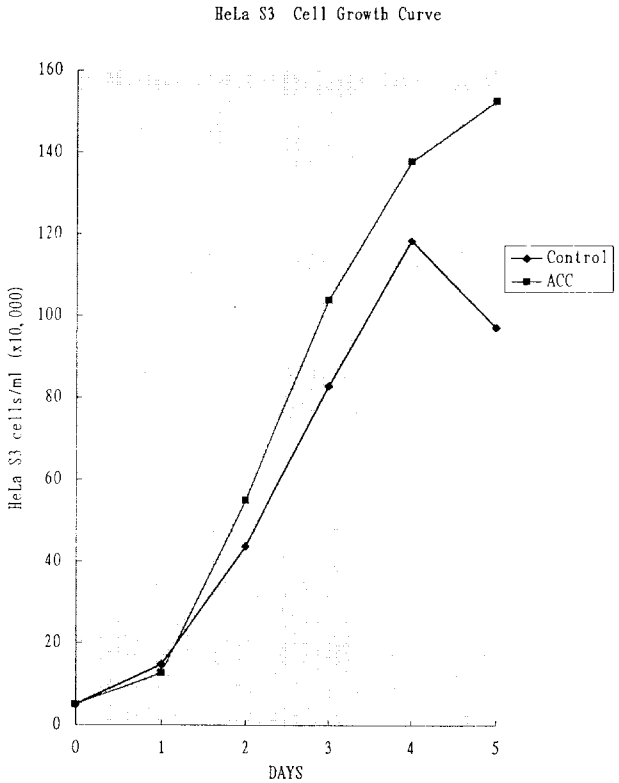

Fig. 3. L929 cell growth curve
initial seed : 5×10^4 cells/ml Liquid Specimen

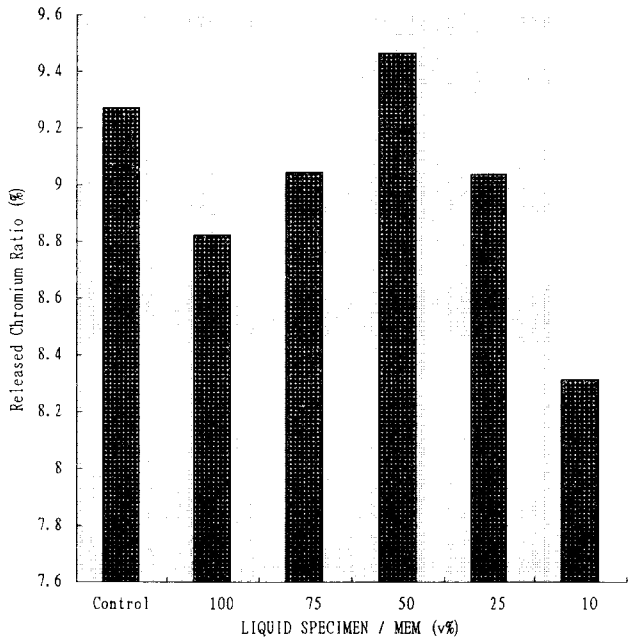

Fig. 4. Released ^{51}Cr from the labelled HeLa S3 cells in the Liquid Specimens

released ^{51}Cr from the labelled cell demonstrates similar reliability and sensitivity as much as the agar diffusion method[10, 11]. If the ^{51}Cr labelled cells face any inhibition on cellular activity in media, the dysfuctional cells are going on cellular degeneration cascade, and ^{51}Cr are released from the cells to media.

In this experiment, a less ^{51}Cr was detected from the composite dissolved liquid specimen than from the control (Fig. 4), though there was no significant statistical difference between them ($p > 0.05$). From the 50 v% liquid specimen, the released ^{51}Cr quantity was a little higher than the negative control. But the result could be acceptable as non-toxic to cells according to the ASTM criteria[10], which specifies that to be recognized as "no cytotoxicity", the liquid specimen should reveal less than 10% on the released ^{51}Cr ratio. All the groups released less than 10% ^{51}Cr.

CONCLUSION

As *in vitro* cytotoxicity tests for an artificial bone substitute composite, growth rates of the incubated HeLa S3 and L929 cells were investigated. Significant statistical differences were observed when the initial seeding cell numbers were controlled to 5×10^4 cells/ml MEM. The cells incubated in the composite dissolved MEMs revealed higher growth rates than those in the control MEM. On released amount of ^{51}Cr from the labelled HeLa S3 cells, all the liquid specimen were acceptable as no cytotoxic on cellular level.

ACKKNOWLEDGMENT

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=국문초록=

체내 매입후 경시적으로 분해되면서 재생골조직에 의해 치환되는 인공골복합체를 제조하고, 복합체가 세포활성에 미치는 영향을 조사하였다. 복합체시편을 세포배양액에 넣고 1주일동안 37℃에서 배양한 다음, 사람자궁경부암유래 HeLa S3세포와 쥐피하L929세포를 복합체가 용해된 세포배양액에서 5일간 배양하여 세포성장율을 비교하여 세포독성을 조사하였다. 한편 HeLa S3세포를 배양중인 배양액에 $Na_2{}^{51}CrO_4$를 첨가하여 HeLa S3세포에 ^{51}Cr를 표식한 다음, 용해된 ^{51}Cr의 양을 γ-counter를 이용하여 측정하였다.

세포성장정도의 측정에서는 HeLa S3 세포 및 L929 세포 모두가 특이한 세포독성을 발견할 수 없었으며, 복합체가 용해된 세포배양액내의 표식된 HeLa S3 세포로 부터 용해된 ^{51}Cr량을 측정한 결과, 세포활성을 저해하지 않은 것으로 관찰되었다.