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Preparation of bioelectret collagen and its influence on cell culture *in vitro*

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Abstract Collagen extracted from pigskin was corona-charged negatively by a specifically designed device. Different charging voltages, temperatures and times were applied to prepare collagen bioelectret. The decline of the surface potential of the bioelectret under different treatment was then determined. The data showed that the surface potential was markedly varied with the charging conditions. The optimal values of three parameters for charging collagen coatings were defined as follows: voltage, 8 KV; temperature, 40°C; time, 25 min. Treatment of the bioelectret with distillated water, or saline solution (0.9%) or culture medium induced a sharp decrease of the surface potential. In addition, we investigated the effects of the charged collagen on cell growth and intracellular calcium level of three types of cultured mammalian cell lines, including Chinese hamster ovary CHO cells, human cervix uteri tumor HeLa cells and human promyelocytic HL-60 cells. Cell growth and the intracellular calcium level were determined by MTT reagent-based assay and a fluorescent probe Fura-2, respectively. The results showed that negatively charged collagen stimulated the growth of CHO or HL-60 cell line but inhibited the growth of HeLa cell line. Furthermore, after attaching to the charged collagen, the intracellular calcium level of CHO cells increased, while that of HeLa cells decreased. Thus we proposed for the first time that collagen bioelectret could differentially modulate the growth of different cells, by an unknown mechanism that probably involves a role of intracellular calcium.

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1. Introduction

Electrets refer to dielectric materials that have net charge or polarization status for quasi-permanent time. As a kind of functional material, electrets have been studied thoroughly and applied in several areas due to their unique properties. Bioelectret, one of the most important ramifications of electrets, is especially noticeable because of its close relation with life activities. Electrets' effect exists in many tissues [1,2] and most important biopolymers such as protein, polysaccharide and some poly-nucleotides [3]. Some bioeletret materials have been used to stimulate the growth of tissues such as bone, nerve, etc [4,5]. Collagen, the main structural protein in the connective tissues of living beings, has been used as a biomedical material due to its good biocompatibility and mechanical properties. Natural collagen has many dipolar groups and shows typical electret behavior, thus, it can in principle be induced into the electret status.

In one of our previous reports, the thermally stimulated current technique was proven effective enough to prepare collagen bioelectrets [6]. In order to study the effects of collagen bioeletctrets on cell behavior in vitro, we have designed a novel device which could negatively charge *in situ* collagen coatings on the bottom of a four-well culture plate. With the utilization of the device, four samples can be simultaneously charged under the same conditions, and this device has been proven very helpful for reducing experimental error and suitable for biological tests of electrets [7].

Some papers have reported that fluoroethylene propylene electret and other polymeric piezoelectric substrates could influence neurite outgrowth in vitro [8,9]. In the present experiments, different charging voltages, temperatures and times were carried out to find out the optimal parameters for preparing collagen bioelectret via corona-charging method. Then the influence of the bioelectret on the growth of three types of cultured mammalian cell lines was investigated, as well as on the intracellular calcium level. We discovered that collagen bioelectret could differentially modulate the growth of different cell types, possibly by altering the intracellular calcium concentration.

2. Materials and methods

2.1. Cell lines and cell culture

Three mammalian cell lines used in the present experiments included CHO, HeLa and HL 60 cell lines, deriving from Chinese hamster ovary, human tumor of cervix uteri and human promyelocytic leukemia, respectively. All cell lines were cultured in DMEM medium, supplemented with 15% fetal calf serum, 100 U/ml penicillin and 100 μg/ml streptomycin at 37°C in 5% CO_2 atmosphere.

2.2. Preparation of collagen bioelectret

Collagen was extracted from pigskin as described before [10]. It was then dissolved at the concentration of 1.0 $mg.ml^{-1}$ in 0.02 M acetic acid. Collagen coatings were formed on the bottom of each well of 4-well culture plates (Nunclon) by drying overnight collagen solution at room temperature and under UV irradiation. Corona charging method was employed to treat collagen coatings in order to make negative charge deposit on their surfaces as shown in the previous paper [7]. The surface potential was examined by a SD-8303 surface potential meter.

2.3. Charge decay assays

To investigate the charge decay of the charged collagen coatings kept in normal or dried air, the surface potential was measured every 24 hours for 20 days. In the other experiments, charged collagen coatings, immersed with distillated water, saline solution (0.9%) and DMEM (GIBCO) medium respectively, were kept in an atmosphere of 37°C and 5% CO_2 produced by an incubator and the surface potential was examined every 10 minutes.

2.4. Cell growth determination

Cells in logarithmic phase were suspended in culture medium to a concentration of 5×10^4 cells/ml. 0.5 ml suspension was added to each well of 4-well plates with charged or non-charged collagen coatings. After growing for 48 hours, cells were counted by the method of MTT reagent (3(4,5-dimethylthiazol-2-yl) 2,5-diphenyl terazolium bromide)-based colorimetric assay [11]. MTT reagent can be converted into blue formazan only by viable cells. Lysis of cells produced a blue solution, the optical density value (OD value) of which was measured with a MODEL 550 microplate reader in a dual wavelength way with the measurement wavelength 450 nm and the reference wavelength 655 nm. The obtained data were statistically analyzed using Student's *t*-test.

2.5. Measurement of the intracellular calcium concentration

The intracellular calcium was measured by the method described by Sun et al [12] with a little modification. The suspension of cells, loaded with Fura-2 (Molecular probes Co.) via a 45-min incubation in the dark at 37°C, was prepared at the concentration of $1\text{-}2\times10^6$ cells/ml culture medium, and added into each well of the 4-well plates coated with charged or non-charged collagen. Since it took less than 30 min for cell attachment, the concentration of intracellular calcium ($[Ca^{2+}]_i$) was measured at time points of 30 min and 45 min after addition of cells. The calcium imaging and $[Ca^{2+}]_i$ measurement was conducted on the MiraCal imaging system, supplied by Life Science Resources (Cambridge, UK). Images were captured by a Nikon Diaphot 200 inverted fluorescence microscope that was coupled to a Mira 1000 TE low light level CCD camera and a computer workstation and quantitatively analyzed with the MiraCal version 2.3 software program (Life Sciences Resources, Cambridge, UK). Since the peak excitation wavelength of the fluorescent Fura-2 shifts from about 380 to 340 nm upon Ca^{2+} binding, the ratio of 510 nm emission intensities at two wavelengths may provide a measurement of free $[Ca^{2+}]_i$. The light source was a 75 W xenon, equipped with a dual monochromator system. $[Ca^{2+}]_i$ was calculated using the well-known ratio equation. Ratio (340 nm/380 nm) of 0.32, 1.82 and 2.25 were estimated to correspond to $[Ca^{2+}]_i$ of 0 nM, 1000 nM, and 2000 nM respectively.

3. Results and dicussion

3.1. Collagen bioelectret preparation

Collagen bioelectret was prepared via the corona-charging method by a novel specific instrument designed by our lab.

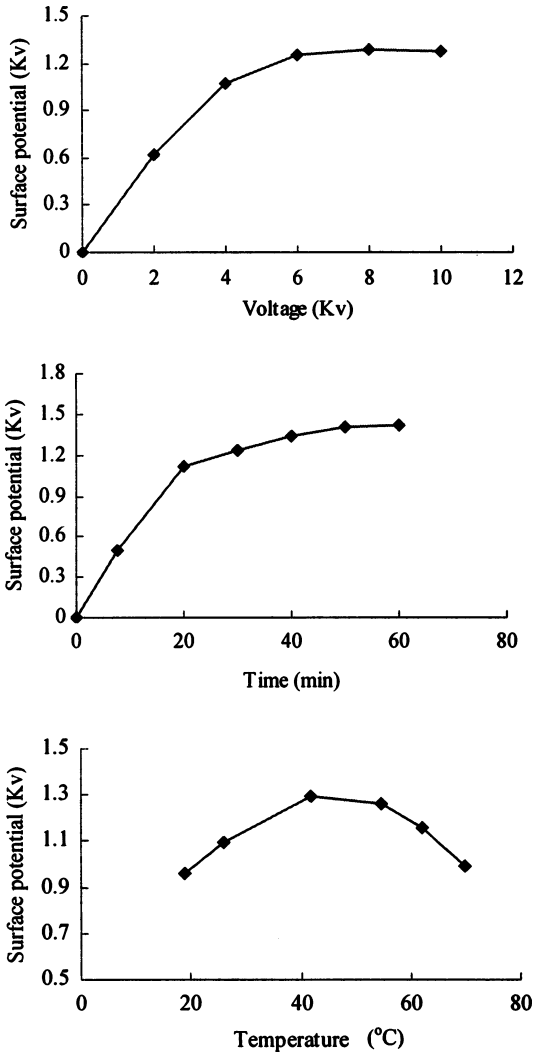

Fig. 1 The influences of charging voltage (upper), time (middle) and temperature (lower) on the surface potential of collagen bioelectret prepared via corona-charging method. Upper: time, 20 min; temperature, RT. Middle: voltage, 8 KV; temperature, RT. Lower: voltage, 8 KV; time, 20 min.

This device turned out to be multi-functional and suitable for studying the biological effects of electret in our previous work [7]. Figure 1 shows the influences of charging voltage, time and temperature on the surface potential of collagen bioelectret. The surface potential increased linearly when the applied voltage was less than 4 KV, then slowly, and finally reached a steady level when the applied voltage was bigger than 8 KV. The influence of the charging time on the surface potential was very similar to that of the applied voltage. However, the charging temperature influenced the surface potential quite differently. The surface potential went up linearly with the rising charging temperature lower than 40°C, and went down over 40°C. Therefore, the optimal values of three parameters for charging collagen coatings were defined as follows: voltage, 8 KV; temperature, 40°C; time, 25 min. Collagen could be negatively charged to the highest surface potential of about 1.2 KV by the above device under the optimal condition.

3.2. Charge decay analysis

Figure 2 shows the difference in the charge decay between collagen bioelectrets stored in normal and dried air. Under both circumstances, the surface potential decreased in a similar way. The surface potential decreased very fast during the first 5 days with a 40–50% loss on the 5th day, then decreased slowly during the following 10 days and kept a steady level later, remained about 40% of the initial level in the end. However, storage in dried air appeared to be able to slow down slightly the rate of charge loss, which implied that humidity might play a role in the inactivation of the bioelectret. This was supported by the fact that the charge loss became much greater when the bioelectret was placed in aqueous solutions. As shown in Figure 3, the surface potential of the bioelectret declined to about 10–25% of the original after treated with different liquid media including distilled water, saline solution (0.9%) and DMEM culture medium for only 40 min. Moreover, it was also found that DMEM medium caused the highest charge loss among the three media, while

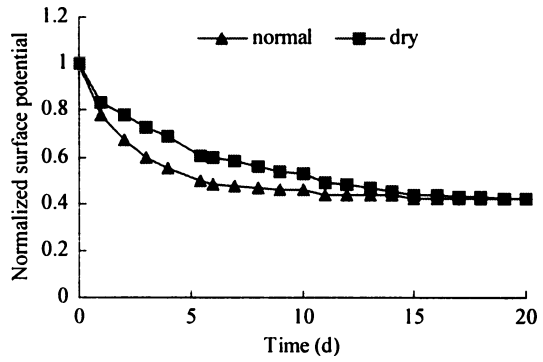

Fig. 2 Charge decay of collagen bioelectret under normal or dried condition.

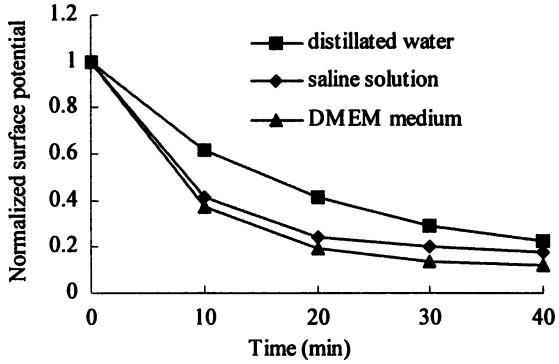

Fig. 3 Charge decay of collagen bioelectret under various aqueous conditions.

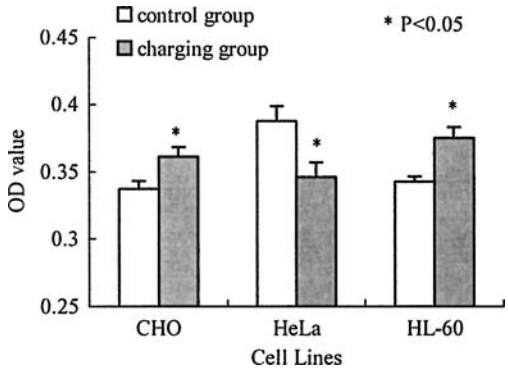

Fig. 4 Influences of collagen bioelectret on the growth of different types of cell lines.

distilled water the lowest (Figure 3). These data, altogether with those reported on the decay of positively and negatively charged fluoroethylene propylene (FEP) electret in aqueous solution by Makohliso et al. [8], suggested that ionic strength be another factor to influence charge stability within the bioelectret.

3.3. Effect of collagen bioelectret on cell growth

Figure 4 shows the influences of charged collagen coatings with initial surface potential of 1.2 KV on the growth of different types of cell lines including CHO, HeLa and HL60 cells. Various cell lines responded in different ways to the bioelectret coatings. Both CHO and HL60 cells could be stimulated to grow, while the growth of HeLa cells was significantly inhibited ($p < 0.05$).

Among the cell lines tested, CHO cells were fibroblast-like cells, HeLa cells were epithelium-like cells and HL60 cells were lymphocyte-like cells. Furthermore, CHO and HeLa cells must adhere to the substratum then begin to proliferate, while HL60 cells grow and divide in a suspension way. The present data showed that all three cell lines could respond to collagen bioelectrets, indicating that interaction between cells and the charged matrix may be tight contact dependent or independent. In the case of tight contact independent way, there might have an unknown extracellular mediator (chemical or physical) which plays a role in signal transduction.

3.4. Effect of collagen bioelectret on the intracellular Ca^{2+} concentration

So far calcium has been regarded as a key modulator of cell growth [13]. As an attempt to explain why different cell lines behaved differently on the bioelectret coatings, we examined the influence of the bioelectret on the intracellular calcium concentration ($[Ca^{2+}]_i$) of CHO and HeLa cell lines.

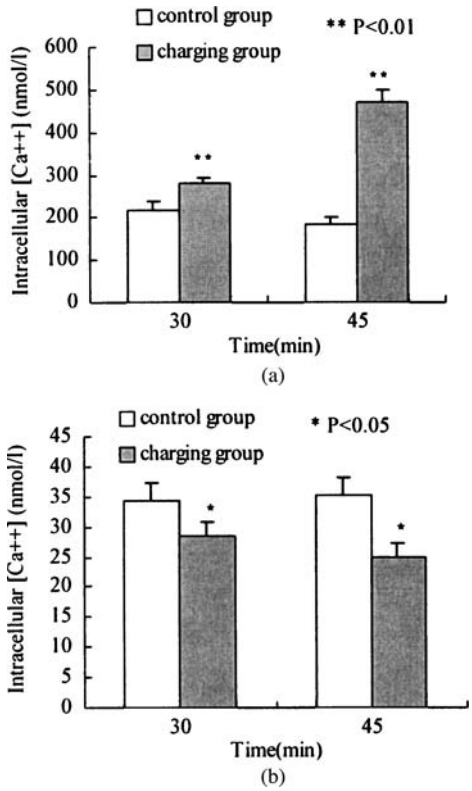

Fig. 5 Effects of collagen bioelectret on the intracellular calcium levels of CHO (a) and HeLa (b) cell lines.

Because $[Ca^{2+}]_i$ measurement must be taken after cell attachment and it took no more than 30 min for both types of cells to attach to collagen matrix, we determined $[Ca^{2+}]_i$ at time-points of 30 min and 45 min after the addition of cell suspension to collagen coatings. The results were shown in Figure 5. At both time-points, the bioelectret was found to increase $[Ca^{2+}]_i$ of CHO cells and decrease $[Ca^{2+}]_i$ of HeLa cells, in accordance with the effects of the bioelectret on the growth of the two types of cell lines (Figure 4). Therefore, we propose that calcium is an intercellular mediator for the bioelectret effects.

Several papers have reported that charged surfaces can affect the adhesion and morphology of a variety of cell types [7–9, 14–15]. To our knowledge, this is the first time to deal with the influence of the same charged substrate on the growth of different cell types. Unexpectedly, the preliminary data presented in this study showed that the influence varied with the cell type. Experiments including more cell types and charged substrates should be done to get a further defined conclusion. While the mechanism(s) by which charges influence cell growth remained unclear [15], intensive investigate should be made to elucidate the mechanism(s) by which various cells act differently on a certain charged surface. We hypothesized that the diverse response of intracellular calcium might be a part of the mechanism(s). Efforts are being made to explore the upstream regulation of intracellular

calcium level in cultured cells growing on the surface of charged collagen.

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References

1. E. FUKADA and I. YASUDA, *Jpn. J. Appl. phys.* **3** (1964) 117.
2. S. B. LANG, *Nature.* **112** (1966) 704.
3. S. MASCARENHAS, in Electrets, Topics in Applied Physics, edited by G. M. SESSLER, (Springer Verlag, Berlin, 1987) p. 321.
4. L. C. KLOTH, J. A. FEEDAR, J. R. CORSETTI and S. E. LEVINE, 11th Mtg. of Bioelectrical Repair and Growth Society Transaction. Vol. XI, Scottsdale, AZ, (1991).
5. J. JIANG, Z. H. WANG, L. HAO and Z. F. XIA, 9th International Symposium on Electrets. ISE 9, Shanghai, China. (1996).
6. S. SUN, T. X. KOU and H. S. ZHU, *J. Appl. Polym. Sci.* **64** (1997) 267.
7. X. L. YANG, B. S. LI, J. W. GU, S. SUN and H. S. ZHU, in Proceedings of the 20th Annual International Conference of the IEEE Engineering in Medicine and Biology Society ("Biomedical Engineering Towards The Year 2000 and Beyond"), Hong Kong, 1998, edited by H. K. CHANG & Y. T. ZHANG, **20(6)** 1998, p. 2970.
8. S. A. MAKOHLISO, R. F. VALENTINI and P. AEBISCHER, *J. Biomed. Mater. Res.* **27** (1993) 1075.
9. R. F. VALENTINI, T. G. VARGO, J. A. GARDELLA JR and P. AEBISCHER, *Biomaterials.* **13** (1992) 183.
10. W. WANG, Y. F. HU and J.Z. XUE, *J. Biomed. Eng.* **8** (1991) 309.
11. A. DEKKER, C. PANFIL, M. VALDOR, H. RICHTER, C. MITTERMAYER and C. J. KIRKPATRICK, *Cells and Materials.* **4** (1994) 101.
12. F. Z. SUN, J. HOYLAND, X. HUANG, W. MASON and R. M. MOOR, *Development.* **115** (1992) 947.
13. K. P. LU and A. R. MEANS, *Endocr. Rev.* **14** (1993) 40.
14. R. KAPUR, J. LILIEN, G.L. PICCIOLO and J. BLACK, *J. Biomed. Mater. Res.* **27** (1996) 133.
15. Q. QIU, M. SAYER, M. KAWAJA, X. SHEN and J.E. DAVIS, *J. Biomed. Mater.* Res. **42** (1998) 117.