Unique cytological behavior of MC3T3-E1 osteoblasts on H₂O₂-modified C/C composites *in vitro*

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ABSTRACT MC3T3-E1 osteoblasts were cultured on H₂O₂-modified and unmodified carbon/carbon (H-C/C and C/C) composites for one week in order to evaluate differences in cell adhesion, spreading, and proliferation. The results indicated a certain degree of enhancement in the cell adhesion capability of osteoblasts cultured on H-C/C samples. Cellular morphologies after cell adhesion were observed via scanning electron microscopy (SEM), which showed that the cells adhered more closely and spread more widely on the H-C/C sample surface. However, no cell appeared in several multiple and continuous types of minor pores on both the C/C and H-C/C surfaces. In addition, two unique situations were observed on the H-C/C samples: an outline change of the osteoblasts was observed when the cells spread across some minor pores, and the cells entered and adhered well in some larger pores.

Keywords: carbon/carbon composites, MC3T3-E1 osteoblasts, H₂O₂ modification, surface wettability

INTRODUCTION

Carbon-based materials are ubiquitous, and the major substances of living organisms are carbon compounds [1]. Large varieties of biomaterials are based on or contain elemental carbon. With continuous progress in the design and manufacturing processes of carbon materials, those with suitable structures and mechanical properties are becoming important biomaterials [2,3]. Carbon/carbon (C/C) composites are unique materials consisting of a carbonaceous matrix and reinforcing carbon fibers. As a type of pure carbon material, C/C composites overcome the strength and toughness problems present in previous pure carbon materials, while incorporating the biological advantages of elemental carbon [4,5]. In addition to being popular high-temperature structural materials for

aircraft and aerospace applications, C/C composites are also potential biomaterials for bone replacements owing to their favorable mechanical properties and good biocompatibilities. For example, they can be prepared as artificial long bones, spine, hip prostheses, knee fracture fixation, intramedullary nail, and bone plates [6–8]. Most of these studies have achieved favorable results; however, C/C composites remain bioinert and hydrophobic. These disadvantages make such materials unable to conduct or induce bone regeneration. Moreover, in some cases, the wear debris of C/C composites would release into the surrounding tissues during or after an implant operation [9–11].

To improve the bioinert and hydrophobic surface of C/C composites, we have successfully prepared a surface with a dense and continuous collagen film [12,13]. H₂O₂ modification was applied on the C/C composites before the film preparation. As a result, several kinds of oxygen-containing functional groups were formed on their surfaces, and the contact angles were reduced from $92.3 \pm 0.8^\circ$ to $65.3 \pm 8.4^\circ$. As is well known, wettability is considered an important criterion that can dictate the biocompatibility of implant materials [14]. Wei *et al.* [15] studied the relationship between surface wettability and cell compatibility. They concluded that although cells could adhere to the substrates with varying surface wettability, those with a more hydrophilic surface had more cells attached as compared to those with a hydrophobic surface. In addition, the cell spreading speed and area on the substrate with a lower contact angle was faster and larger than those on the substrate with a higher contact angle.

The same effect may also occur on the surface of H₂O₂-modified C/C composites. However, C/C composites have certain unique features. As is well known, the

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appearance of micro-pores in C/C composites is inevitable. Although we could select the C/C samples with higher densities to reduce the volumes and amounts of micro-pores, the influences of irregular micro-pores on cell adhesion and spreading cannot be completely eliminated. In addition, mechanical processing on the C/C composites would also generate micro-pores. Therefore, the cell attachment and spreading details on a hydrophilic surface of the C/C composites still need to be confirmed. Hence, in our study, MC3T3-E1 osteoblasts were cultured on C/C and H₂O₂-modified C/C (H-C/C) composites in order to detect the improvement of cell attachment and proliferation caused by H₂O₂ modification. The different details of the cytological behaviors of MC3T3-E1 osteoblasts on the C/C and H-C/C samples were also observed using scanning electron microscopy (SEM).

EXPERIMENTAL SECTION

C/C composites

C/C composites were prepared through a chemical vapor infiltration (CVI) process in our laboratory. The carbon preforms used were carbon fiber-needled felts, and natural gas was used as the carbon source for infiltration [16]. The sample density was between 1.70 and 1.75 g cm⁻³. Prior to surface modification and coating preparation, each sample was polished with No. 400, No. 800, No. 1200, and No. 1500 SiC sandpapers, rinsed with distilled water, then cleaned ultrasonically in acetone, and dried in a drying cabinet [17].

Surface modifications

C/C samples were immersed in a 50 mL 30% H₂O₂ solution, which had been maintained at 100°C in a thermostat water bath. After a 4-hour treatment, the modified C/C samples were taken out, then washed with deionized water three times, and dried in a vacuum environment. The oxidized C/C samples were considered as H-C/C samples [12].

In vitro cell culture and evaluation

Prior to use, all the C/C and H-C/C samples were sterilized with an autoclave. MC3T3-E1 cells were obtained from Shanghai Institutes for Biological Science (SIBS) of Chinese Academy of Sciences (CAS). The cells were cultured in minimal essential medium- α (MEM- α) containing 10% fetal bovine serum (FBS). After reaching 80% confluence, the adherent cells were washed, trypsinized, counted, and resuspended to seed on the samples.

To determine the cellular adhesion and proliferation, each sample was seeded with 700 μ L of the cell suspension, corresponding to a cellular concentration of 2×10^4 mL⁻¹ in medium (MEM- α with 10% FBS), and cultured for one week at 37°C. These specimens were used for evaluation with the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) test on days 1, 3, 5, and 7, respectively. For detection, the samples were moved into a new 24-well cell culture plate without medium, and 100 μ L MTT (Amresco, USA) solution (5 mg mL⁻¹) and 900 μ L new medium was added to each well, followed by incubation at 37°C for 4 h. Subsequently, the newly added medium with MTT was removed from each well, and 1 mL dimethyl sulfoxide (DMSO, Amresco, USA) was added to the wells. The plate was shaken for 15 min, and 200 μ L DMSO was moved from each well into new wells in a 96-well cell culture plate, followed by absorbance detection at 490 nm on a microplate reader (Bio-Tek Instruments, USA).

Cellular morphology observations

To observe the cellular morphology of osteoblasts cultured on the C/C and H-C/C samples, 700 μ L of the cell suspensions with a concentration of 5×10^4 mL⁻¹ were seeded on the samples. The cell-containing samples were then incubated at 37°C and fixed with glutaraldehyde 24 hours later. After acetonitrile replacement, vacuum drying, and gold sputter coating, samples with cells were observed via SEM (Hitachi S3400, Japan).

Statistical analysis

Statistical analysis was conducted using the statistical software package SPSS 13.0. The statistical differences between the two groups were calculated using Student's *t*-tests. Statistical significance was declared at $P < 0.05$.

RESULTS

In vitro evaluations of H-C/C samples

Fig. 1 shows the cell adhesion and proliferation status of MC3T3-E1 osteoblasts cultured on the C/C and H-C/C samples for one week *in vitro*. The data demonstrated that the osteoblasts adhered to and proliferated well on the C/C and H-C/C samples. The absorbance data also showed that the cells had higher cell adhesion values on the surface of the H-C/C samples than on the C/C samples. Although the improvement caused by surface wettability enhancement was not statistically significant, the H₂O₂ modification did

Figure 1 Cell viability of MC3T3-E1 osteoblasts on the surfaces of C/C and H-C/C samples.

not result in cytotoxicity.

Cellular morphology comparisons of osteoblasts between C/C and H-C/C samples

Cellular morphologies shown in Fig. 2 demonstrate the fine cell adhesion and favorable cell spreading conditions of the osteoblasts on the surfaces of both the C/C and H-C/C samples. Several cells were spread over the trenches caused by the peeling off of the carbon fibers, as well as the cracks and minor pores formed between the fiber and matrix. However, a more comprehensive comparison revealed several differences between the groups with respect to minute details. Compared with the cells on the C/C sample, the osteoblasts on the H-C/C sample seemed much flatter with less pseudopods, while the gaps between the cell and substrate were smaller.

In addition, several isolated situations occurred in these two different substrates. The first situation was the appearance of two not-fully spread cells on the surface of the C/C sample; these cells were clearly in weak condition. The second situation was an outline change of the cell. In particular, a cell was partly covered with a hole on the H-C/C surface in Area 5. However, the same phenomenon was not found in Area 2 on the C/C sample. The third isolated situation was a cell monolayer that formed in Areas 3 and 4 on the H-C/C sample, which demonstrated that the cells appeared confluent on the surface. The last situation was detection of a multiple-trench structure that conspicuously blocked cell adhesion. In Area 6, a cell could partly migrate into the multiple-trench structure; however, adherence of a complete cell could not directly be observed in this kind of trench structure (Area 7).

Unique cell-spreading details of osteoblasts on H-C/C samples

As shown in Fig. 3, several unique cell-spreading details of MC3T3-E1 osteoblasts were observed in various micro-pore areas of the H-C/C sample, which were not observed for the C/C surface. The first situation was that cells migrated into large pores in Areas 1, 2, and 6 of the H-C/C sample. In these areas, the cell fully spread and adhered well to the substrate, although some parts of it migrated into pores and cracks. The second situation was observed in Area 3, in which a cell seemed to be suspending over a large pore in midair. Although this cell was partly connected with the matrix, it still spread well in a satisfactory condition. The last situation was that cells were deeply buried in the large pores in Areas 4 and 5. Although only some small parts of the osteoblasts could be observed in the pores, an

Figure 2 Cell morphologies of MC3T3-E1 osteoblasts cultured on (a) the C/C sample and (b) the H-C/C sample.

Figure 3 Cell morphologies of MC3T3-E1 osteoblasts cultured in some large pores on the H-C/C samples.

assumption could still be inferred that these cells were cultured well.

DISCUSSION

In our previous research, numerous hydroxyl, carboxyl, and carbonyl functional groups were formed on the surface of an H-C/C sample [12]. The improvement in surface wettability enhanced the adhesion numbers of MC3T3-E1 cells on the H-C/C surface.

Examination of the morphologies of MC3T3-E1 cells on the C/C and H-C/C samples raised some puzzling questions. Why could the micro-pores in the C/C sample be observed directly in SEM photographs? That is, why were these micro-pores not covered by cells? In one specific situation observed in Area 7 (Fig. 2b), no cell appeared, and the multiple-trench structure of the substrate was exposed. We considered that this situation would not have occurred if the cells had been cultured in a culture plate at the same cell seeding density. To determine how this might have happened, we consulted the relevant literature related to the morphologies of osteoblasts on the C/C composites, which showed that these micro-pores also clearly appeared in previous studies. An inference was drawn that air residue formed in the micro-pores of the C/C and H-C/C samples after the cell suspension fully covered when we con-

sidered the situation of the not-fully spread cells in Area 1 (Fig. 2a). In such areas, air films were formed between the substrates and the medium, which blocked full contact between the resuspended cell and the C/C substrate during the cell seeding operation. We tried to classify the irregular micro-pores in the C/C composites according to size or shape to help explain how these properties might affect cell adhesion; however, the micro-pores were too complex to be classified in this manner. Therefore, we next tried to classify these micro-pores into several categories using the scale of a resuspended MC3T3-E1 cell as a reference standard. We divided the blocking effects into three situations (Fig. 4). In situation I, one cell adhered to the C/C substrate with no obvious pores, and cell spreading was normal. Most of the adhered cells were in this state and then fully spread with extraordinary cytoactivity. In situation II, a cell was surrounded by air films, and only small parts could connect with the substrate. The multiple pores formed influenced cell adhesion, resulting in the not-fully spreading cells (such as the cells in Area 1 of Fig. 2a). The cytoactivity of osteoblasts decreased and the proliferation ability was also delayed. In situation III, residual air in the continuous minor pores blocked the contact between the cell and substrate. In such areas, the cell would have difficulty surviving because it could not be able to attach to the

Figure 4 Schematic diagrams of cell adhesion and spreading processes in diverse situations.

Figure 5 Schematic diagrams of MC3T3-E1 cell spreading across some tiny pores on the C/C and H-C/C samples. (a) Air hidden in the pore of a C/C sample. (b) Cell spread across the pore on a C/C sample. (c) Air hidden in the pore of an H-C/C sample. (d) Cell spread across the pore of an H-C/C sample.

substrate without a connection. The above situations in which residual air hidden in the complex minor pores (situations II and III) on the surface of the C/C composite could prevent cell adhesion and spreading.

Although the blocking effect could not be eliminated by the H_2O_2 modification, the advantages conferred by the oxidation still reduced the impact of the residual air in the

micro-pores. The outline change of MC3T3-E1 cells that was only observed on the H-C/C surface (such as the cell shown in Area 5 of Fig. 2b) could explain the improvement effect in the minor pores. As shown in the schematic drawing in Fig. 5, the cells could spread over the minor pores in both the C/C and H-C/C samples. We thought that the interface of gas and liquid had sunk into the pores

of the H-C/C sample. When the spread of a cell crosses over the minor pore, it would spread along this sunken interface. The outline of the cell in the sunken area changed the visual effect under SEM observation (such as the cell shown in Area 6 of Fig. 2b). This phenomenon demonstrated that the oxidation improved the wettability of the inner surface of the micro-pores. Although the wettability improvement could not eliminate the air in the H-C/C sample, the balance of the gas and liquid interface was nevertheless changed. In addition, we thought that this tiny difference in minor pores would not directly reflect the enhanced cell adhesion numbers on the H-C/C surface. However, in large pores, the wettability improvement would be useful. Cells could deeply penetrate the large pores (such as the cells shown in Area 4 of Fig. 3b and in Area 5 of Fig. 3c). The fine adhesion and spreading states of MC3T3-E1 cells indicated that the resuspended cells could follow the medium entering the large pores. This may directly reflect the enhanced cell adhesion numbers observed in the comparison of the MTT data between the C/C and H-C/C samples.

For the C/C composites, the formation of micro-pores is inevitable. The cellular morphology differences on the C/C and H-C/C samples highlighted the importance of these micro-pores. Therefore, to improve the bioactivity of C/C composites via surface modification in the future, the negative effects caused by the existence of micro-pores must be considered and then minimized.

CONCLUSIONS

Common points and differences in the morphology of MC3T3-E1 cells on the C/C and H-C/C samples indicated that the existence of micro-pores would cause negative effects for cell adhesion. The use of H₂O₂ modification could minimize these negative effects and benefit cell spreading into large pores.

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Author contributions Cao S and Li H designed the experiment; Cao S and Zhang L engineered the samples; Cao S cultured the cells and carried out the tests; Cao S wrote the paper with support from Lu J. All authors contributed to the general discussion.

Conflict of interest The authors declare that they have no conflict of interest.

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MC3T3-E1成骨细胞在H₂O₂改性碳/碳复合材料表面特殊的细胞学行为

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摘要 本文评估了MC3T3-E1成骨细胞在碳/碳复合材料和H₂O₂改性的碳/碳复合材料表面的细胞黏附、增殖和细胞铺展的细节差异. 结果表明, 成骨细胞的黏附率在改性的碳/碳复合材料表面有一定的提高. 通过对细胞形貌的扫描电子显微镜(SEM)观察和对比, 两个特殊的细胞形貌使我们意外发现细胞的黏附与碳/碳复合材料表面固有的孔隙有着密切关系. 一个是没有细胞在碳/碳复合材料表面可见的孔隙处, 另一个是成骨细胞在跨越孔隙时出现轮廓的变化. 最后我们还发现, 成骨细胞可以黏附到改性碳/碳复合材料的内部.