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Original article

Cinnamic aldehyde inhibits proliferation and invasion in a well-defined 3-dimensional culture of human cutaneous melanoma cells in tissue engineered-skin

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Background: Tissue-engineered skin can be used not only to cover and repair skin damage, but also as a model to test the efficacy of drugs and cosmetics.
Objective: To establish a 3-dimensional (3D) culture model of skin melanoma invasion using A375 melanoma cells in vitro for studying melanoma and with which to conduct preliminary evaluation of therapeutic drugs. Here we evaluated the efficacy of cinnamic aldehyde to inhibit tumor cell growth in our 3D model of malignant melanoma.
Methods: Melanoma cells A375 were inoculated onto the surface of tissue-engineered skin and cultured at the air–liquid interface. On day 5, cinnamic aldehyde (20 μ□) was added to the culture medium. Skin samples cultured for different days were stained with hematoxylin and eosin and observed using transmission electron microscopy. Immunohistochemical staining of E-cadherin, proliferating cell nuclear antigen (PCNA), and matrix metalloproteinase (MMP)-9 was conducted separately.
Results: A large number of A375 cell clumps had invaded the deep dermis by day 15. The tumor cells formed clumps through the desmosomes and connected with the surrounding fibroblasts through cell junctions. While the expression of E-cadherin was lost in the tumor cells, expression of MMP-9 and PCNA increased with increasing depth of invasion. Cinnamic aldehyde inhibited the proliferation and invasion of melanoma cells in the 3D culture model. Expression of MMP-9 and PCNA significantly decreased in melanoma cells in the model treated with cinnamic aldehyde.
Conclusion: The 3D culture model successfully retains the biological proliferation and invasion characteristics of the malignant melanoma cells and can be used as a system to study further the biological characteristics of malignant melanoma and to evaluate the efficacy of drug treatment. Cinnamic aldehyde and compounds of its class may prove useful treatments for patients with advanced melanomas.

Keywords: Cinnamic aldehyde, invasion, melanoma, proliferation, tissue-engineered skin, 3-dimensional culture

Abbreviations

2D = 2-dimensional
3D = 3-dimensional
DED = de-epidermized acellular dermis
DMEM = Dulbecco's Modified Eagle Medium
EGF = epidermal growth factor
HE = hematoxylin and eosin
KSFM= keratinocyte serum free medium
MMP = matrix metalloproteinase
PCNA = proliferating cell nuclear antigen
TEM = transmission electron microscopy
TIMPs = tissue inhibitors of metalloproteinases

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Engineered skin tissue has been in development for nearly 30 years [1]. In general, the engineering first involves the generation of an epidermal substitute with a single-layer structure [2, 3]. The dermal substitute is then developed into a 3D culture [4]. In the final part of the process the bilayered skin substitute becomes a viable model [5] with the inoculation of human fibroblasts into the extracellular matrix and keratinocytes onto the dermal surface. After cultivation, these cells form a skin substitute with both epidermal and dermal layers. Tissue-engineered skin can be used, not only to cover and repair various skin damage, but as a model to test the efficacy of drugs and cosmetics [6-10].

Melanoma usually originates from skin and is the most aggressive form of skin cancer with a high mortality [11]. The invasive growth of malignant melanoma is the crucial factor in determining its clinical prognosis. To understand the biological characteristics of cell proliferation and invasion by malignant skin melanoma, and to search for a safe and effective treatments are key issues that need to be resolved. Using tissue-engineered skin as a 3D model of the invasion of malignant melanoma in vitro has great potential for further studying the biological characteristics of malignant melanoma and evaluating the efficacy of drugs. In addition, it may also provide a basis and justification for the use of 3D culture models of other tumors in vitro. Cinnamic aldehyde is an α,β-unsaturated aldehyde that is FDA-approved for use in foods. Cinnamic aldehyde has been proven to induce cell death in several types of tumors including melanomas [12]. It has been demonstrated that cinnamic aldehyde inhibits melanoma cell proliferation and invasion in two-dimensional (2D) culture [13]. In this study, we tested the therapeutic efficacy of cinnamic aldehyde in our 3D model of malignant melanoma. This 3D model closely resembles the microenvironment of melanomas in vivo. Hence, it can be used to evaluate the efficacy of potentially therapeutic drugs accurately and effectively. Our results show that cinnamic aldehyde inhibits melanoma cell proliferation and invasion in the 3D model.

Materials and methods

Culture of seed cells

Epidermal keratinocytes and dermal fibroblasts were isolated from the foreskins of healthy donors (6 to 18 years old) who visited the dermatology clinic of our hospital. The keratinocytes were digested with trypsin, and fibroblasts were digested with collagenase II (Gibco Invitrogen, Carlsbad, CA, USA). Second to fourth-passage keratinocytes and fifth to tenth-passage fibroblasts were used in further experiments. Keratinocytes were cultured in KSFM (Gibco Invitrogen). The fibroblasts were cultured in DMEM medium (Gibco Invitrogen) supplemented with 10% bovine calf serum (Lanzhou Minhai Bioengineering, Lanzhou, Gansu, China). Human malignant melanoma cell line A375 cells were purchased from the Cell Bank of the Chinese Academy of Sciences (Shanghai, China) and cultured in high-glucose DMEM medium (Gibco Invitrogen) containing 10% fetal bovine serum (Gibco Invitrogen).

Construction of tissue-engineered skin

A matrix containing type I collagen of rat tail tendon (obtained from rat tail tendon at a concentration of 20 mg wet weight per mL), chitosan (Sigma, St. Louis, MO, USA), DMEM, chondroitin sulfate, hyaluronic acid, and elastin (Sigma) was mixed well in an ice bath, and adjusted to a pH of 7.2-7.4. The fibroblasts were suspended in this matrix at the density of 1×10^5/mL. After solidification of the matrix at room temperature, keratinocytes and A375 cells (in the ratio of 5:1) were inoculated onto the gel. The basal medium was DMEM, which contained 10 ng/mL EGF (Sigma), 5 μg/mL insulin (Novo Nordisk, Bagsvaerd, Denmark), 0.4 mg/mL hydrocortisone (Sigma), and the concentration of glucose in the medium was adjusted to facilitate cell growth and differentiation. The tissue-engineered skin was submerged in the medium and cultured for 3 days. It was then cultured at the air–liquid interface for another 12 days after contraction of the skin. On day 5, cinnamic aldehyde at a concentration of 20 μ was added to the culture medium. The culture medium was changed every two days. Each treatment was performed in triplicate.

Transmission electron microscopy (TEM) observation

Fifteen-day-old cultured skin samples were cut into 0.5 × 0.1 cm long strips, fixed in glutaraldehyde, embedded, and sectioned. After positioning under a light microscope, the sections were mounted onto copper grids and observed by TEM.

Hematoxylin and eosin (HE) staining and immunohistochemistry

Tissue-engineered skin samples cultured for 5, 10, and 15 days were fixed overnight in 4% buffered formaldehyde, paraffin-embedded, sectioned, and stained with HE. On days 10 and 15, the cinnamic aldehyde-treated skin samples (20 μM from day 5) were also fixed and stained with HE. The immunohistochemical staining of E-cadherin, PCNA, and MMP-9 was conducted separately. The average gray value of the immunohistochemical preparations was quantified with a HMIAS-2000 High-resolution Medical Color Image Analysis System (Wuhan Champion Imaging Technology, Wuhan, China). The expression level was inversely proportional to the average gray value. Five random high-power fields of each PCNA-positive tissue section were counted for PCNA-positive cells out of 100 tumor cells. The percentage of positive cell was calculated.

Statistical analysis

Results are presented as means±SD of three independent experiments. Analysis was completed using the two-tailed Student's t test. Values of $p < 0.05$ were considered statistically significant.

Results

HE staining

HE staining showed that the melanoma cells formed clumps in the dermis and from there invaded downward. The invasion became deeper with the extension of culture time. After 5 days of culture, the tumor cells were distributed in the superficial layer of the dermis (**Figure 1A**). Extensive invasions into the deep dermis can be observed after 10 and 15 days of culture (**Figure 2A and C**). In contrast, melanoma cell clumps were smaller and closer to the epidermal–dermal junction in cinnamic aldehyde-treated skins (**Figure 2B and D**).

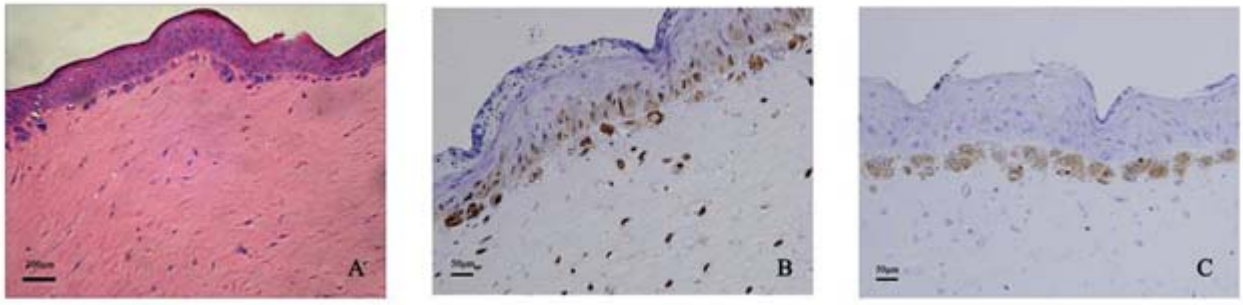

Figure 1. **A:** A375 cells in the superficial dermis of tissue-engineered skin after 5 days culture (HE × 200) **B:** Expression of PCNA of A375 cells in tissue-engineered skin after 5 days culture (SP × 400). **C:** Expression of MMP-9 of A375 cells in tissue-engineered skin after 5 days culture (SP × 400)

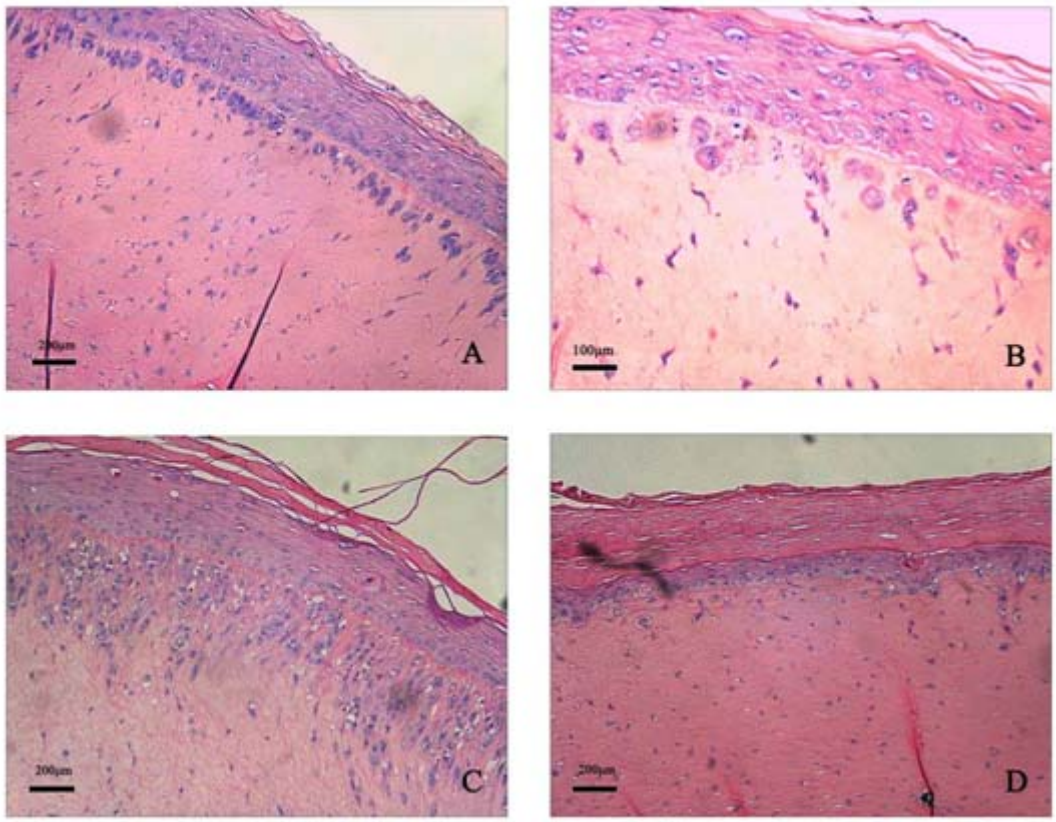

Figure 2. **A:** A375 cells form clumps in the dermis of tissue-engineered skin after 10 days culture (HE × 200). **B:** A375 cells form clumps in the dermis of cinnamic aldehyde- treated (20 μM from day 5) tissue-engineered skin after 10 days culture (HE × 400). **C:** A375 cells form clumps in the dermis of tissue-engineered skin after 15 days culture (HE × 200). **D:** A375 cells form clumps in the dermis of cinnamic aldehyde-treated (20 μM from day 5) tissue-engineered skin after 15 days culture (HE × 200)

TEM observations

The melanoma cells grew well within the dermis, and presented atypically shaped nuclei and abundant organelles (**Figure 3A**). The tumor cells formed clumps through the desmosomes and connected with the surrounding fibroblasts through cell junctions (**Figure 3B–C**).

Immunohistochemical staining

Melanoma cells in the dermis stopped expressing E-cadherin, while the expression of PCNA and MMP-9 increased with the deepening of tumor invasion (**Figure 1B and C, Figure 4A and C, Figure 5A and C**), while melanoma cells in cinnamic aldehyde-treated skins faintly expressed PCNA and MMP-9 (**Figure 4B and D, Figure 5B and D**). The levels of expression were significantly different among cells after 5, 10, and 15 days of culture (**Figure 6A**). The rates of PCNA-positive cells also exhibited significant differences among the 5-, 10-, and 15-day cultures (**Figure 6B**). The differences were also present in cells in the control and cinnamic aldehyde-treated skin groups (**Figure 6B–C**).

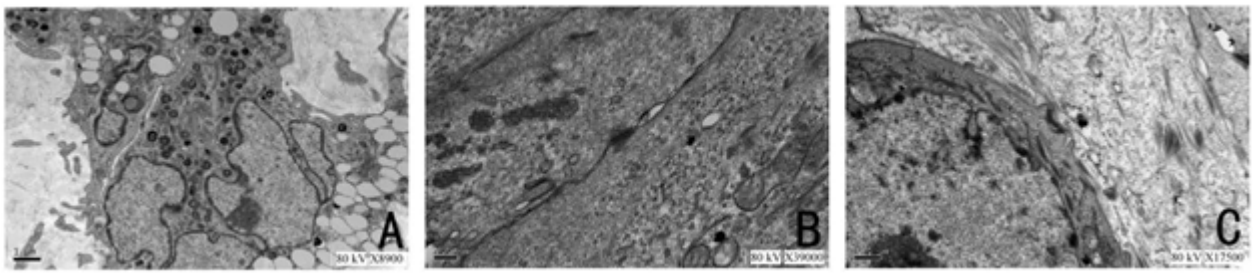

Figure 3. A: A375 cell in dermis. **B:** Desmosomes between two A375 cells. **C:** Junction between A375 cell and fibroblast

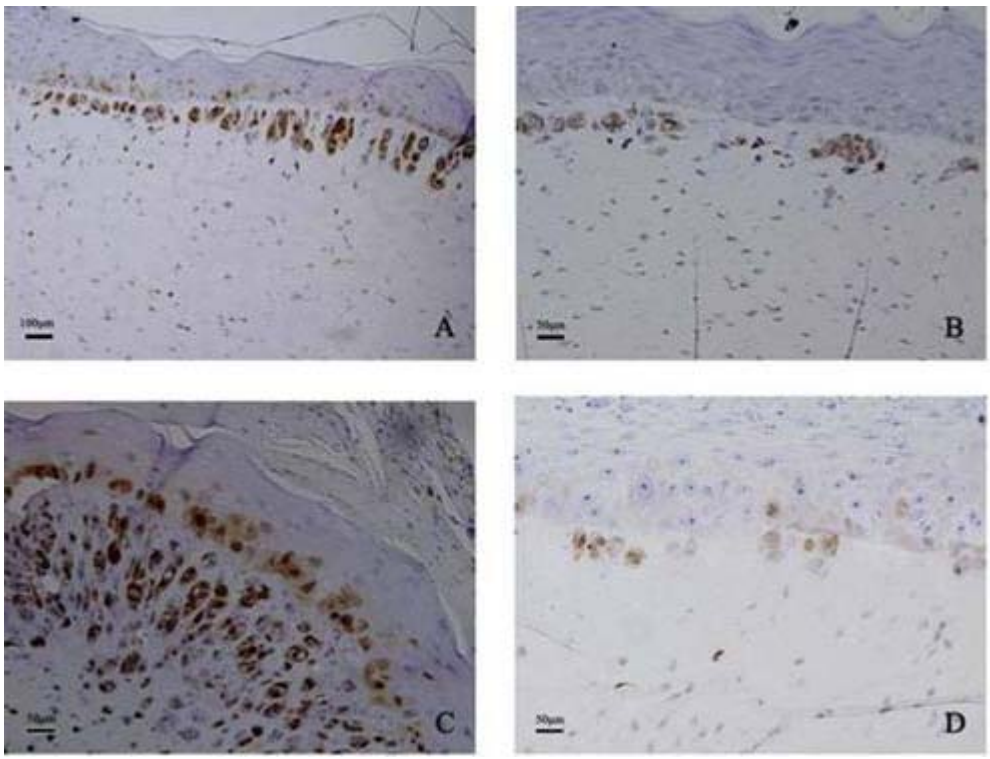

Figure 4. A: Expression of PCNA of A375 cells in tissue-engineered skin after 10 days culture (SP × 200). **B:** Expression of PCNA of A375 cells in cinnamic aldehyde- treated (20 μM from day 5) tissue-engineered skin after 10 days culture (SP × 400). **C:** Expression of PCNA of A375 cells in tissue-engineered skin after 15 days culture (SP × 400). **D:** Expression of PCNA of A375 cells in cinnamic aldehyde-treated (20 μM from day 5) tissue-engineered skin after 15 days culture (SP × 400)

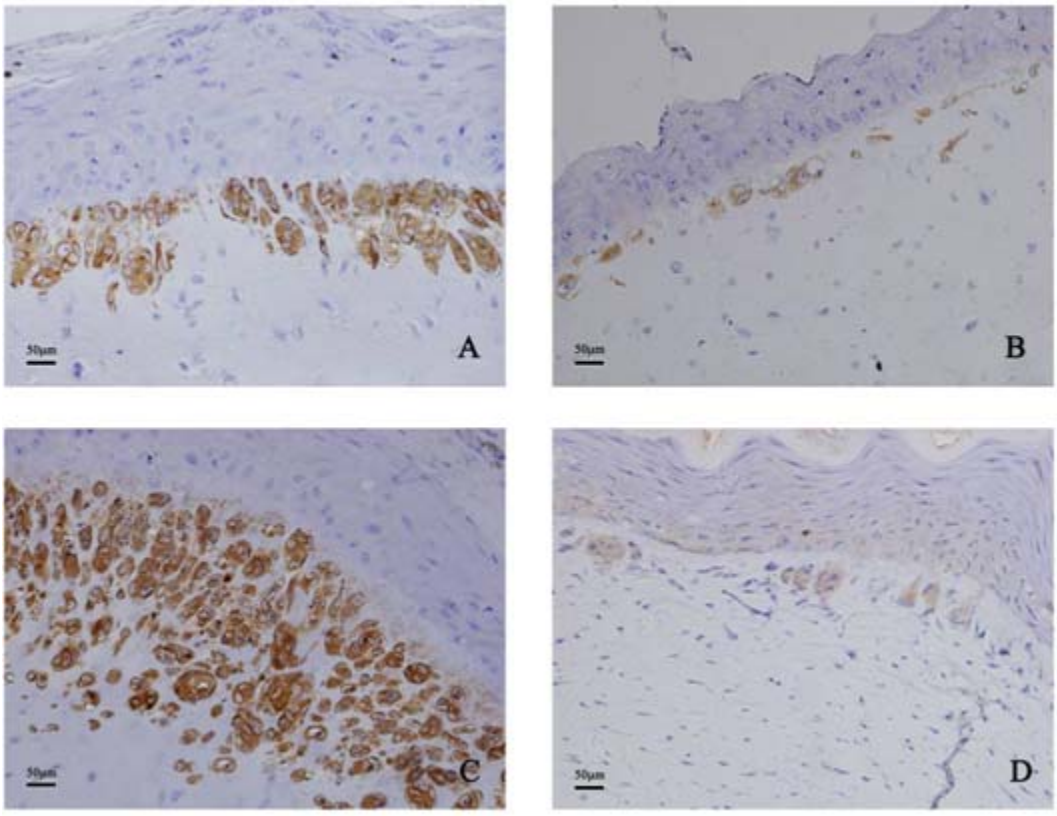

Figure 5. **A:** Expression of MMP-9 of A375 cells in tissue-engineered skin after 10 days culture (SP × 400). **B:** Expression of MMP-9 of A375 cells in cinnamic aldehyde-treated (20 μM from day 5) tissue-engineered skin after 10 days culture (SP × 400). **C:** Expression of MMP-9 of A375 cells in tissue-engineered skin after 15 days culture (SP × 400). **D:** Expression of MMP-9 of A375 cells in cinnamic aldehyde-treated (20□M from day 5) treated tissue-engineered skin after 15 days culture (SP × 400)

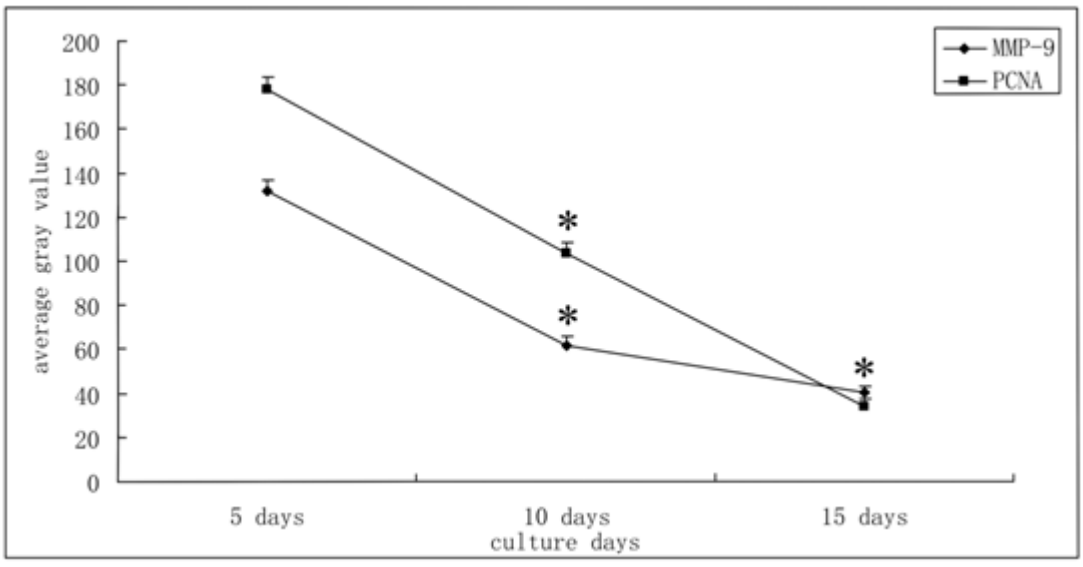

Figure 6 A. The average gray value of MMP-9 and PCNA of A375 cells in tissue-engineered skin in different culture days. *p <0.05 compared to 5 days.

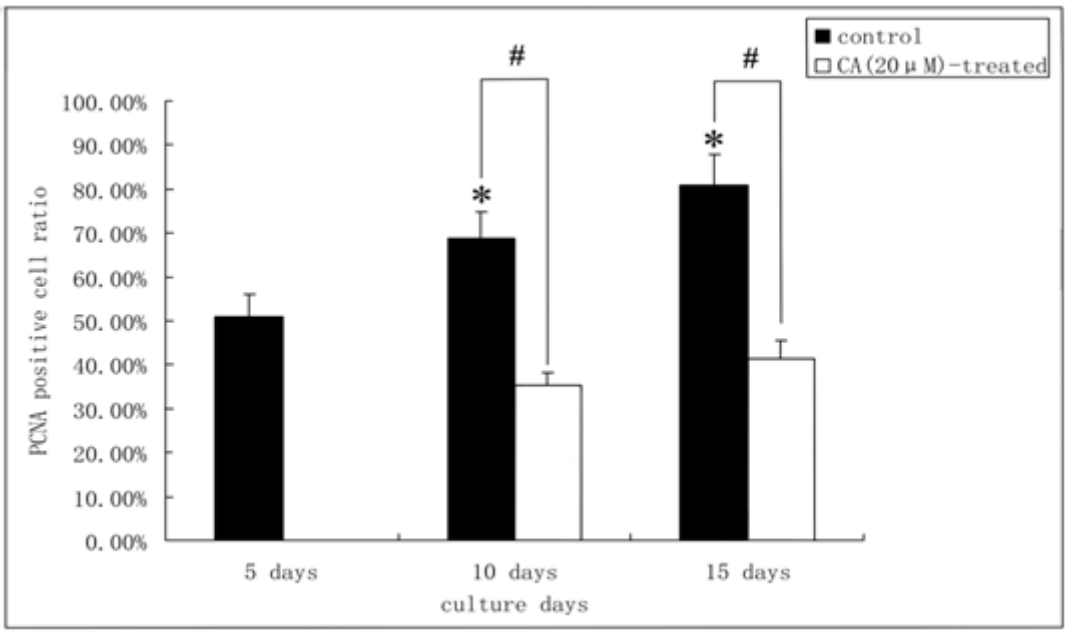

Figure 6 B. The proportion of PCNA positive cells to A375 cells in control and cinnamic aldehyde- treated (20 μM from day 5) tissue-engineered skin in different culture days. *p <0.05 compared to 5 days. #p <0.05 compared to control.

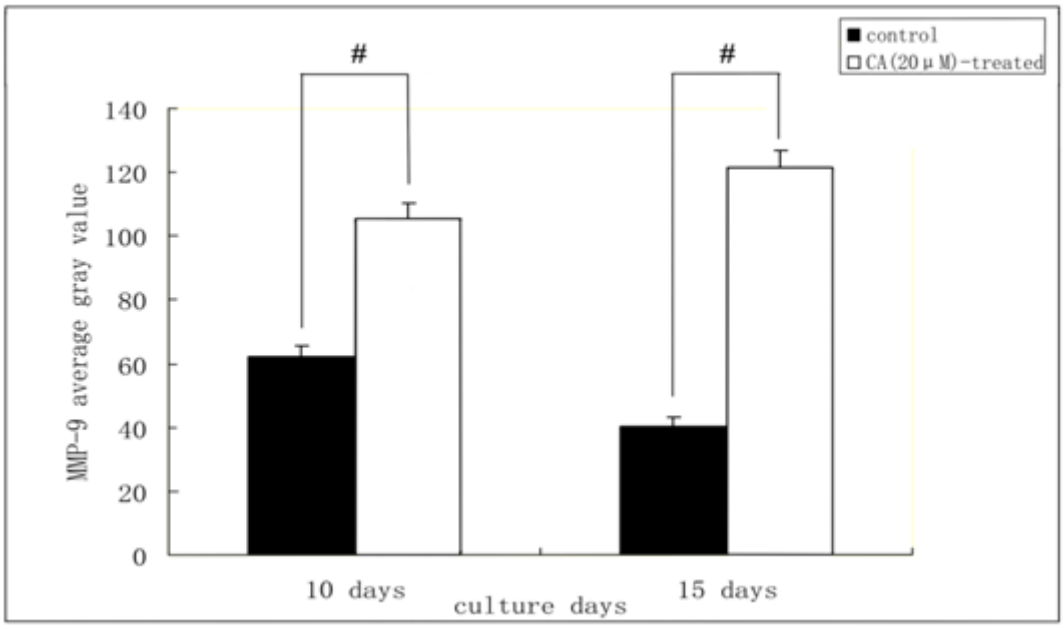

Figure 6 C. The average gray value of MMP-9 of A375 cells in control and cinnamic aldehyde-treated (20 μM from day 5) tissue-engineered skin in different culture days. #p <0.05 compared to control.

Discussion

Malignant melanoma is the most invasive and fatal of the skin cancers. To understand its biological characteristics better, a variety of melanoma cell lines have been isolated, cultured, and used in medical research [14, 15]. A375, an invasive melanoma cell line, has been used in the present study. The current research in the field of melanoma studies is mainly focused on 2D culture of tumor cells, and animal models with tumor inoculation or spontaneous tumors, all of which have limitations. Results from work with animal models are often biased because of differences between species, between the animal body and the human-originated melanoma cells [16]. 2D cell culture is a major tool in cell biology research, and 2D culture of A375 cells in vitro reproduces some of the biological characteristics of melanoma cells in vivo. However, it cannot be used to observe the processes involved in cell–cell connections, the signal transduction between cells and their extracellular matrix, and the changes of gene expression during tumor cell invasion because of the restriction of growth environment (the common drawback of the 2D culture of tumor cells) [17]. Some researchers have used de-epidermized acellular dermis (DED) [18] or collagen gels [19] as scaffolds in skin models of melanoma. This study has successfully established a 3D culture model of myeloma using A375 cells and tissue-engineered skin technology. In this 3D culture model, the extracellular matrix in the dermis is composed of type I collagen, chitosan, and glycosaminoglycans. The electrostatic interactions and crosslinking in the dermal matrix strengthens the skin dermal structure. This makes it more stable and closer in form to the growth environment of melanomas in vivo and provides a new tool with which to study the biological characteristics of melanomas in vitro. During the development of the 3D culture model of malignant melanoma in vitro, we have found that culture at the air–liquid interface is most suitable for the growth of melanoma cells, which is consistent with a previous report [20]. Because nutrition is highly demanded for the rapid growth of melanoma cells in vitro, the authors have adjusted the concentration of glucose in the culture medium to provide the necessary nutrients to inoculated melanoma cells on the dermis directly, without affecting the growth of epidermal keratinocytes and dermal fibroblasts. After 5 days of culture at the air–liquid interface in the improved medium, the epidermis was well differentiated and the dermal fibroblasts also grew well. The dermal invasion by melanoma cells can be observed, and gradually became deeper with the extension of culture time. After 15 days of culture, a large number of melanoma clumps were seen inside the dermis. TEM showed that A375 cells grow well in the dermal matrix and possess enriched cell processes, organelles, and endoplasmic reticulum. Melanoma cells in the clumps were connected by desmosomes.

Cinnamic aldehyde is an active constituent extracted from cinnamon and possesses diverse biological function [21]. The beneficial effects of cinnamon extract on melanoma cells in two-dimensional culture have been reported previously [13]. However, the efficacy of this extract on melanoma in 3D culture is unknown. Our findings show that cinnamic aldehyde is able to moderate the growth and invasion of melanoma cells in 3D tissue-engineered skin. In our 3D model, melanoma cells interact with keratinocytes, fibroblasts, and the extracellular matrix. The communication contributes to the cell differentiation and molecular signal transduction between epithelial and mesenchymal cells.

We observed that cinnamic aldehyde apparently reduces the growth and proliferation of melanoma cell clumps and decreases the invasion depth of melanoma cells in tissue-engineered skin.

The invasion and metastasis of malignant tumors is a complex biological process. Penetration of the basement membrane and extracellular matrix is a prerequisite for tumor cells to migrate to other tissues and organs. Therefore, proteolytic enzymes that can degrade the extracellular matrix are necessary for tumor cell invasion and metastasis. MMPs are a class of Zn^{2+} and Ca^{2+}-containing proteolytic enzymes, playing important roles in angiogenesis and tumor invasion and metastasis. One of the major abnormalities of the molecular biology of malignant melanoma is the dysregulation of the expression of MMPs and tissue inhibitors of metalloproteinases (TIMPs) [22, 23]. Secretion and activation of MMPs promote the tumor cells to degrade the basement membrane and achieve profound invasion. Among the MMPs, MMP-9 mainly degrades type IV collagen and is one of the major proteins related to tumor invasion. Inhibition of the MMP-9 expression attenuates tumor invasion and metastasis [24]. The gradual increase of MMP-9 expression with increasing depth of melanoma cell invasion seen in this study indicates that the expression of MMP-9 is positively related to the invasion of tumor cells. The decreased expression of

MMP-9 in melanoma cells in tissue engineered skin treated with cinnamic aldehyde suggests that cinnamic aldehyde markedly inhibits melanoma invasion. Loss of expression of the transmembrane glycoprotein E-cadherin is one of the factors in determining the highly invasive characteristics of tumor cells. E-cadherin not only plays a role as adhesion molecule, but also prominently involves the invasion and metastasis of melanoma cells through catenin-mediated cell signaling [25]. Inhibiting the expression of E-cadherin promotes melanoma metastasis [26, 27]. Keratinocytes regulate the growth and proliferation of melanocytes through E-cadherin-mediated cell connections. A375 cells inside the tissue-engineered skin do not express E-cadherin and thereby separate from the epidermal keratinocytes and invade the dermis.

The expression of PCNA is closely related to DNA synthesis [28] and can be used as an indicator to evaluate the state of tumor cell proliferation, which is associated with various aspects of the development, grading, staging, recurrence, and metastasis of the tumors [29, 30]. High expression of PCNA is related to the invasion of malignant melanoma [31]. By prolonging melanoma cell growth and increasing the depth of invasion in the tissue-engineered skin, the proportion and intensity of PCNA expression increases gradually. The decrease of PCNA positive cells in cinnamic aldehyde treated melanoma cells in tissue-engineered skin indicates that cinnamic aldehyde inhibits the proliferation of melanoma.

Conclusion

In conclusion, our data demonstrate that the 3D culture model of malignant skin melanoma established in the present study exhibits good stability and is highly controllable. The model exhibits the biological characteristics of proliferation and invasion of malignant melanoma cells. It can therefore be used as a model system to study the biological characteristics of malignant melanoma cells and to evaluate the efficacy of potentially therapeutic drugs. We found that cinnamic aldehyde significantly reduces the growth of melanoma and prevents its invasion into the dermis in this 3D model. On the basis of this model, we believe that cinnamic aldehyde may potentially play a beneficial role in the treatment of patients with advanced melanoma.

Acknowledgements

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