

The effect of mitomycin-c in keloid fibroblast cultures

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ABSTRACT

Keloid occurs due to hyperactivity of keloid fibroblast (KF) in proliferation, migration, collagen deposition, together with low rates of collagen degradation. These are under the responsibility of TGF- β . Mitomycin C (MC) is used for treating keloid by a topical application during surgery at the level of 0.02% to 0.08%. Unfortunately, the lowest effective level of MC for keloid has not been determined yet. We aimed to determine the lowest effective level of MC in the suppression of KF activities. Various levels of MC diluted in growth medium were administered on KF that were isolated from six patients. After 24 hours and 72 hours of incubation, cellular proliferation, collagen deposition, cellular migration and level of TGF- β , were analyzed. Application of 120 μ M MC on KF culture for 24 hours could significantly reduce TGF- β production from 1265.74 ± 274.81 pg/mL to 265.17 ± 12.20 pg/mL; proliferation index from 100% to $84.01 \pm 12.91\%$; inhibit cellular migration to $64.38 \pm 3.66\%$; but reduce collagen depositions from 100% to only $91.13 \pm 10.19\%$. The lowest MC level is on 30 μ M or equal with 0.001%. In conclusion, the lowest level of MC can suppress the activities of KF is 0.001%. Moreover, due to low activity in inhibiting collagen deposition, MC would be better as an adjuvant drug for keloid surgery.

ABSTRAK

Keloid timbul karena proliferasi, migrasi, dan sintesis kolagen oleh fibroblas keloid (FK) secara berlebihan diikuti dengan rendahnya degradasi kolagen. Semua itu terjadi karena pacuan TGF- β . Olesan mitomycin C (MC) 0,02% sampai 0,08% digunakan untuk mengobati dan mencegah kekambuhan keloid yang dioperasi. Sayangnya, dosis terendah yang masih efektif belum pernah diteliti. Penelitian ini bertujuan untuk menentukan kadar terendah MC yang masih efektif untuk mengobati keloid. MC dilarutkan dalam medium pertumbuhan dalam berbagai kadar dan diberikan pada biakan FK yang diisolasi dari material keloid enam orang pasien. Setelah inkubasi 24 jam dan 72 jam, proliferasi, migrasi, timbunan kolagen dan kadar TGF- β , dianalisis dan dibandingkan. Pemberian 120 μ M MC pada biakan FK ternyata dapat menurunkan produksi TGF- β dari $1265,74 \pm$

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274,81 pg/mL menjadi 265.17 ± 12.20 pg/mL; indeks proliferasi dari 100% menjadi $84,01 \pm 12.91\%$; menghambat migrasi sampai $64,38 \pm 3.66\%$, tetapi daya hambat timbunan kolagen hanya dari 100% menjadi $91.13 \pm 10.19\%$. Kadar MC terendah yang masih efektif adalah 30 uM atau setara dengan 0.001%. Kadar MC di bawah itu tidak lagi efektif. Kesimpulan, kadar MC terendah yang masih efektif sebesar 0,001% dan karena daya hambat timbunan kolagen yang rendah, MC sebaiknya digabung dengan operasi keloid.

Keywords: keloid fibroblast – mitomycin-c - TGF- β – deposition – prolifiration

INTRODUCTION

Keloid is a fibroproliferative benign tumor that found only on human skin with characteristics in persistent overgrowth of fibrous tissues during wound healing leading to the formation of over scar tissues more than original wound size, and can invade surrounding normal tissue as a crab-claw of keloid lesion.^{1,2} This overgrowing is due to keloid fibroblast (KF) proliferation and producing collagen excessively³⁻⁵ together with low activity of extracellular matrix metalloproteinase (MMP).^{6,7} KF also have a characteristic of mesenchymal stem cells and can migrate to surrounding normal tissues to produce enlargement of fibrous tissues.⁸ Moreover, one of various growth factors that have an important role in keloid behavior is transforming growth factor- β (TGF- β).⁹ This growth factor can induce Wnt/ β -catenin signaling,¹⁰ and over-expression of Wnt is parallel with collagen deposition in both keloid tissues and KF culture.^{11,12} Wnt/ β catenin signaling pathway is not only responsible for collagen production but it also has an important role in inducing transformation of human dermal microvascular endothelial cells to become KF.¹²

Mitomycin-C (MC) is an anti-cancer commonly used for bladder cancer and others, including as the preventing agent for pterygium by topical application intra-operatively.¹³ Interestingly, the target of

this agent is pterygial body fibroblasts that produce fibrotic tissues under stimulation of connective tissue growth factor (CTGF) initiated by TGF- β .¹⁴ Similar with it, MC is also used in keloid lesion by topical application intra-operatively to suppress KF activities. Talmi *et al*¹⁵ apply MC to the wound of keloid excision and resulting in the complete disappearance of keloid lesion. MC can prevent keloid recurrence up to 80% when applied topically to the base of shave-removing keloid surgery.¹⁶ The side effects of topical application of 0.02% MC (equal with 598 uM) among patients with pterygium is low, mild, and reversible.¹³ Application of MC intra-operatively is ranged from 0.02% to 0.08% (equal with 2393 uM), but whether 0.02% as the lowest effective level of MS is still unknown. In order to know the lowest level of MC in topical-application for keloid surgery, we reported the effect of various low level MC on KF culture, therefore, the best level of MC in keloid surgery may be determined.

MATERIAL AND METHODS

Isolation and culture of KF

The core of keloid materials was obtained from six patients underwent keloid debulking surgery with informed concerns. About 2 cm³ of each material was thinly sliced into 2-3 mm³ pieces and cultured by explants method in Dulbecco's Modified Eagle's Medium

(DMEM, Gibco®, USA) containing 10% fetal bovine serum (FBS, Gibco®,USA) and 1% penicillin/streptomycin (Gibco®, USA) at 37°C and 5 % CO₂. The spindle-shaped cells that outgrown from the explants then were subcultured until passage 4.

Experiments

Stock MC-preparation

MC (molecular weight = 334.33) was purchased from Biochem Pharmaceutical Industry Ltd., Mumbai, India under license

from Kyowa Hakko Kogyo, Japan. After dissolving 10 mg MC in 25 mL sterile water (molarity = 1.1964mM), further dilution with DMEM was performed until stock MC in the level of 600 uM. Referring to the findings of Wang *et al.*,¹⁷ on human dermal fibroblasts, we began to treat KF with 120 uM MC.

Design and treatment

All of the experiments were then conducted in the following scheme:

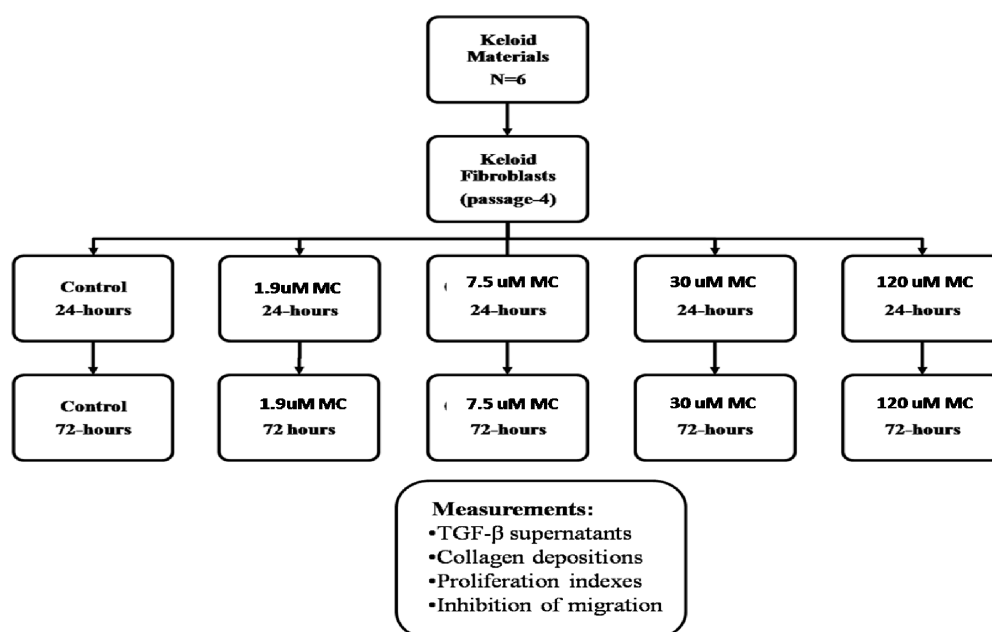


FIGURE 1. Preparation and measurement scheme

The experiment was carried out with KF reseeding in wells of 200 uL cell suspension containing 103 cells/mL. After 24 hours of incubation to let cells attached on the bottom of wells, various treatments carried out in accordance with a written scheme on above. All of the procedures were in triplicate.

Measurement of variables

Proliferation index

Cellular viabilities were measured using MTT {-(3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide purchased from MP biomedical-France} assay and optical density (OD) of formazan blue produced by living cells and were determined using spectrometer at 570 nm of wavelength. All of the ODs from the control group was

considered as 100% of the proliferation index, therefore, the proliferation index of various treated groups was counted as: (OD of treated group / OD of paired control) x 100 %.

Ability to deposit collagen

Collagen deposition was measured by insoluble collagen of Sirius red (purchased from Sigma-Aldrich, Steinheim, Germany) assay according to Taskiran *et al.*,¹⁸ the OD Sirius red-bound collagen represented the amount of insoluble collagen and it was read using spectrometer at 570 nm of wavelength. The OD of the control group was considered as 100%ability to deposit collagen. The ability of various treated groups was counted as: (OD of treated group / OD of paired control) × 100 %.

Inhibition of cellular migration

Cellular migration assay was performed based on method Liang *et al.*,¹⁹ briefly, after serum starvation, all bottom of wells were linearly scratched with the blunt tip of a 32G sterile needle through the center of the well bottom. After cultivation with various media and incubations, the cells were then stained with Meyer's Haematoxylin and microscopic photo images were taken using a Moticam-350 camera (China) in JPG format. Both blue color's pixels of fibroblasts along the scratch line and white color's pixels of empty space can be measured using Adobe-Photoshop. Migration rate was counted as: (blue color pixel of KF along the scratch line / total pixel along the scratch line) × 100%. Inhibition of migration of treated group was counted as

migration rate of control minus migration rate of paired treated group.

TGF-β level

The level of TGF-β in the supernatant of various groups was measured using human TGF-β 1 ELISA kit purchased from Bender MedSystem (Burlingame, USA). The OD of each well was read using spectrometer at 570 nm of wavelength and the level of TGF-β was counted using standard curve obtained from a correlation between various standardized TGF-β with their OD.

Statistics

All data are presented as a mean ± standard error. We used one -ay analysis of variance (ANOVA) followed by LSD to analyze the migration rate data and Friedman test followed by Wilcoxon as post hoc test to analyze the proliferation index and collagen deposition data. The level of statistical significance was accepted at p<0.05.

RESULTS

Application of 120 uM MC on KF culture for 24 hours can reduce: TGF-β production from: 1265.74 ± 274.81 pg/mL to 265.17 ± 12.20 pg/mL; collagen depositions from 100% to 91.13 ± 10.19%; proliferation index from 100% to 84.01 ± 12.91%; and inhibit cellular migration up to 64.38 ± 3.66%. The effect of diluted MC on KF culture both for 24 hours and 72 hours can be observed in the following FIGURES.

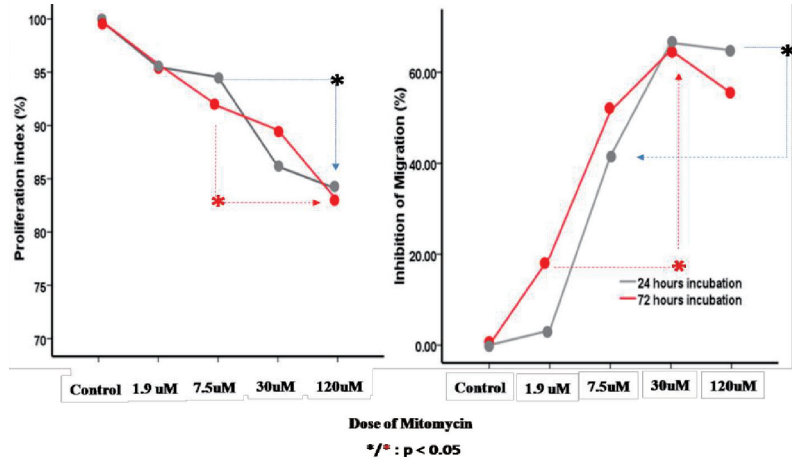


FIGURE 2. Effect of MC on proliferation and cellular migration

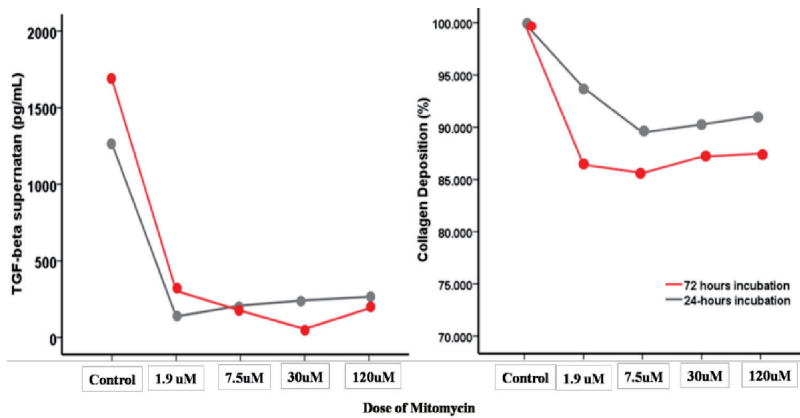


FIGURE 3. The effect of MC on TGF-β and collagen deposition

DISCUSSION

Various keloid treatments, starting from surgery to intralesional corticosteroid and various anti-cancers such as fluorouracil,²⁰ bleomycin,²¹ MC,^{15,16,22} or a combination of them have been performed.²³ MC has been chosen because it can inhibit normal skin fibroblast proliferation, induce fibroblast apoptosis, and regulate intracellular protein expression on mRNA level.¹⁷

Our results showed that 120 uM MC can inhibit proliferation and migration of KF

FIGURE 1. This finding is important because continuously enlargement of keloid lesion is under responsible of high proliferation rate of KF³⁻⁵ and high migration ability to invade surrounding normal tissue.⁸ Application 120 uM MC for 72 hours had no significantly different effect in proliferation index and inhibition of cellular migration than 24 hours. Dilution of 120 uM MC into a quarter, one-eighth and so on indicated that significant different ($p < 0.05$) in KF proliferation and migration was existed on 7.5 uM and below,

both 24 hours and 72 hours of administration. So, it can be concluded that lowest level of MC is on the level of 30 uM or equal to 10.03 ug/mL or equal to 0.001%.

Regulation of KF protein expression by 120 uM MC can be observed in FIGURE 2, as it was indicated by reducing the TGF- β level and collagen deposition. Based on low inhibition of collagen deposition (about 10% of 24 hours of incubation), it seemly indicated that MC as a single drug is not worthy to be used for treating keloid lesion otherwise repeated injections of MC must be given. Based on high inhibition of MC in cellular migration and cellular proliferation on above, it looked that MC administration just has prevention effect in keloid progression. It has been known that extensive enlargement of keloid lesions is due to KF proliferations, collagen depositions, and cellular migrations.³⁻⁵ In order to totally treat keloid lesions, we agree that MC must be combined with keloid surgery that removes the existing fibrous tissue material which richens by collagen bundles, such as previously reported by many authors.^{15,16,22,23} In this combination, unremoving KF and collagen bundles on the surface of debulking lesions of preexisting keloid can be prevented to grow and to be prevented to relapse. The significant different of collagen deposition between 72 hours and 24 hours of application (as it shown by red dot) showed that MC could stimulate collagen degradation as manifestation of MMP activation. Among KF, production of tissue inhibitor of metalloproteinase (TIMP)-2 is high and MMP-2 is low. A study showed that application of IL- β can convert this event to become increasing MMP-2 level together with decreasing TIMP-2 parallel with increasing collagen degradation.²⁴ We suspected that increasing collagen degradation in our experiment was due to MC regulate KF in interleukin- β protein expression. A further

research to clarify this hypothesis should be performed in the future.

So far, there is no any criterion for converting drug level obtained from cell culture to the equal dosage for human in clinical applications. Atashkina *et al.*²⁵ assume that so many variables must be considered before converting those drug's levels in the human body, for example: whether cultured cellular models exhibiting reliable, known and intact biochemical pathways and structural elements are same with keloid lesions. For these purposes, our experiment in two dimensions KF culture had limitation at least in structural elements where excessive collagen bundles before having treatments were not represented in our cells condition. This limitation may affect on effective drug's level that is lower than it should be in keloid lesion. Since there is no representative animal model for keloid study, a further study in three dimensions of organotypic keloid culture must be conducted, so the lowest effective level of MC can be determined. The comparison of 3 dimensions culture's weight and the monolayer culture's weight can be used to determine starting level of MC for further researches.

CONCLUSION

MC is just applicable in preventing recurrence of keloid after having keloid surgery. Moreover, the lowest effective level of MC is 30 uM for a monolayer of KF culture or equal to 0.001% but it must be recalculated in the clinical application of keloid surgery and it depends on the estimation of residual fibrous tissues weight.

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