The effect of combination of triamcinolone acetonide and methotrexate on keloid-fibroblast activity in dermis equivalent

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ABSTRACT

Endra Yustin E. S., Fajar Waskito, Yohanes Widodo Wirohadidjojo - The effect of combination of triamcinolone acetonide and methotrexate on the keloid-fibroblast activity in dermis equivalent

Background: Triamcinolone acetonid (TA) intralesion has been a standard treatment for keloids for many years, due to its effect in inhibiting collagen synthesis and fibroblast proliferation. However, until now the clinical result is unsatisfactory. Keloid flattening is slow and sometimes adverse reactions may occur. Methotrexate (MTX) is a chemotherapeutic agent having an antiproliferating effect which act as an antifolic acid. Because of this effect, MTX is potential to be used in combination with TA for the treatment in keloid. Fibroblast populated collagen lattice (FPCL) was a dermal equivalent usually used for fibroblast activity measurement.

Objective: To understand the inhibition of fibroblast keloid activities of MTX in vitro on FPCL contraction, compared to TA and MTX plus TA.

Methods: This research used simple parallel multigroups experimental study design, and conducted on third passage keloid fibroblast culture, which was isolated from one patient. Fibroblast was cultivated in collagen type 1 from rat tail (FPCL). Keloid fibroblasts was classified into 16 groups, and treated with 5, 10, 20 mM TA, 1.75, 3.5, 7 mM MTX, combination of TA and MTX, and a control negative. FPCL contraction indicating activities of fibroblast was measured using Scion Image software. Mean of FPCL contraction was analyzed using one way analysis of variance (ANOVA).

Results: All treatments could inhibit FPCL contraction until day 2 (p<0.05). The highest inhibition of FPCL was found in combination of TA 20 mM + MTX 7 mM (p<0.05). The treatment that could inhibit FPCL contraction until day 3 was only group MTX 3.5 mM + TA 20 mM. This result indicated that a combination of TA and MTX was stronger in inhibiting keloid fibroblast activities compared with TA and MTX alone.

Key words: keloid fibroblast - growth factors - triamcinolone acetonid - methotrexate - FPCL contraction

ABSTRAK

Endra Yustin E. S., Fajar Waskito, Yohanes Widodo Wirohadidjojo - Efek kombinasi triaminsilon asetonid dan metotreksat terhadap aktivitas fibroblas keloid pada ekivalen jangat


Tujuan: Mengetahui daya hambat aktivitas fibroblas keloid secara in vitro terhadap kontraksi FPCL antara kombinasi TA dan MTX dalam berbagai dosis dengan berbagai dosis TA dan MTX secara tunggal.
Metode: Penelitian ini menggunakan rancangan penelitian eksperimental sederhana paralel multigrup, yang memakai kultur fibroblas keloid passage 3 dari satu penderita keloid. Fibroblas dibiakkan dalam kolagen tipe 1 dari buntut tikus (FPCL). Fibroblas keloid dibagi menjadi 16 grup dan diterapi dengan TA 5, 10, 20 mM, MTX 1,75, 3,5, 7 mM, kombinasi TA dan MTX, dan kontrol negatif tanpa perlakuan. Kontraksi FPCL sebagai indikasi aktivitas fibroblas keloid dinilai dengan Scion Image software. Rerata luas FPCL diuji dengan memakai analisis varians satu jalan (ANOVA).

Hasil: Kelompok MTX dan TA tunggal ataupun kombinasi pada semua dosis dapat menghambat FPCL pada hari pertama dan kedua (p<0,05), hambatan terbesar dijumpai pada kombinasi MTX 7 mM + TA 20 mM (p<0,05). Terapi yang dapat menghambat kontraksi sampai hari ke-3 hanya dijumpai pada kelompok MTX 3,5 mM + TA 20 mM. Hasil penelitian ini menunjukkan bahwa kombinasi TA + MTX lebih kuat menghambat aktivitas fibroblas keloid dibanding TA dan MTX yang diberikan tersendiri.

INTRODUCTION

Keloids are benign hyperproliferative growth of dermal collagen that is usually resulted from excessive tissue response to skin trauma, expands beyond the boundaries of the original injury as it heals and does not regress spontaneously. Keloids are often pruritic, painful, causes cosmetic disfigurement and skin contracture. Keloids may be caused by minor skin wound such as acne, piercing, vaccination, or even a mosquito bite or post-surgery, especially on pre sternal region, superior back of the trunk and posterior region of the neck. Keloid scars mostly affected persons between 10 and 30 years of age, in Blacks and Asians race, with predisposition in individuals with human leukocyte antigen (HLA) B 14, BW 16 and blood group A. The pathology of keloids remains poorly understood. In vitro researches had shown that keloid scars were considered to be a result of abnormal wound healing, due to overproliferation of fibroblast followed by low activities of collagenase that caused overexcessive collagen in the wound. Fibroblast proliferation was stimulated by growth factors and cytokines, such as vascular endothelial growth factor (VEGF), transforming growth factor beta (TGF-b), tumor necrosis factor alpha (TNF-α), and interleukin 6 (IL-6). The most important factor is TGF-b8, followed by VEGF.

Keloid scars exclusively affected Homo sapiens, and that is why no experiments were possible in animal models. The experiment for keloids usually used in vitro model that investigated the organization of collagen fibers by fibroblast in fibroblast populated collagen lattice (FPCL) contraction model. It was composed of cultured fibroblast suspended in collagen lattice isolated from rat tail. This model was a dermal equivalent, which was usually used for fibroblast activities measurement.

Keloid therapy was fraught with varying degrees of success. There was no one modality that was always successful. Various methods had been developed to treat keloid, from pharmacologic treatments such as triamcinolone acetonide (TA), tretinoin, tacrolimus, 5-flourourasil (5-FU), tamoxifen, to surgery. All of these modalities had unsatisfactory results, the keloid flattening was followed by high rates of recurrences. The most commonly used therapeutics for keloid was intralesion TA injection as a single modality or combined with others. A study showed that 10 mM TA caused a significant decrease in the production of TGF-b keloid fibroblast with side effect like severe pain, atrophy, hypopigmentation or Cushing’s syndrome after several injection.

Methotrexate (MTX) is a chemotherapeutic agent having an anti-proliferating effect which act as a folic acid antagonist that usually used to treat breast cancer, acute lymphoblastic leukaemia, psoriasis, rheumatoid arthritis, and kerato-achantoma tumor as a intralesional treatment with no local or systemic adverse effects from the therapy.

As an immunosuppressant, MTX might cause suppression of TNF-α, IL-6, TGF-b, and also had anti-angiogenesis effect, which decreases the level of VEGF. Muzaffar and Tonkin reported success in treating keloid with oral low dose MTX combined with surgery and no recurrences. However, the combination of MTX with TA in keloid derived fibroblast proliferation as well as in collagen synthesis had not been published.
**METHODS**

**Keloid dermal fibroblast primary cultures**

Primary cell lines of keloid dermal fibroblasts were established from operating room specimens. The keloid tissue was taken from deltoid region. A standard explant method was used to establish the primary fibroblast cultures from the operative specimens. This explant method was performed using sterile technique under a laminar flow hood. After all of the subcutaneous tissue was removed with a sterile scalpel blade, the gross specimens were minced into small fragments in a petri dish. The specimens underwent antimicrobial treatment with Dulbecco’s phosphate-buffered saline (PBS) solution with 5% penicillin/streptomycin/amphotericin (PSA). Minced fragments measuring approximately 1 mm were then placed into tissue culture flasks containing 5 ml of explant tissue culture media (40% fetal calf serum in Dulbecco’s modified eagle medium with 1% PSA). These flasks were then stored in a humidified incubator containing a 5% CO₂ atmosphere at 37°C. The explant-containing flasks were examined daily under light microscopy for the outgrowth of fibroblasts from the tissue fragments. Until light microscopy demonstrated such fibroblast outgrowth, explant tissue culture media was changed daily. Once fibroblast outgrowth approached confluence, the tissue fragments were removed. The fibroblasts were then trypsinized with 0.05% trypsin and subcultured into culture flasks containing 10 ml of primary culture media (10% fetal calf serum in Dulbecco’s modified eagle medium with 1% PSA). All flasks were stored in a humidified incubator containing a 5% CO₂ atmosphere at 37°C. Primary culture media was changed twice a week. Successive cultures were passed at confluence. Experiments were performed using passage 3 keloid fibroblasts.

**Preparation of fibroblast-populated collagen lattices**

Collagen lattices were prepared from type 1 collagen extracted from rat tail tendons in 0.1% (vD v) acetic acid. A working solution was constituted by diluting the collagen to 2 mg/ml on the day of the experiment. Keloid fibroblast was trypsinized, resuspended in medium and counted using a haemocytometer. A collagen–keloid fibroblast suspension was prepared in DMEM and divided into 16 groups, then treated with 5, 10, 20 µM TA, 1.75, 3.5, 7 µM MTX, combination of TA and MTX, and a control negative. Fibroblast-populated collagen lattices with treatments were seeded into the wells of 24-well plates at density of 6 × 104 cells/ml. Plates were kept in an incubator with 5% CO₂ at 37°C.

**Macroscopic evaluation of the gel contraction**

The area of collagen gel populated with fibroblasts was photographed with a digital camera (Olympus SLR E.500) at day 0, 1, 2 and 3 after the experiment had begun, the gel area was calculated with Scion Image software. The percentage of gel contraction in each interval studied was calculated with the following formula:

\[
\text{Percentage of contraction} = \frac{(A_1 - A_2)}{A_1} \times 100
\]

where: \(A_1\) is initial gel area and \(A_2\) is the area at the observed intervals.

**Statistical Analysis**

The gel contraction data were analyzed using ANOVA and were expressed as mean percentage of contraction and standard deviation. Posthoc test used Tukey HSD with \(p<0.05\).

**RESULTS**

The mean inhibition of FPCL contraction between treatment and control groups were shown as a graphic (FIGURE 1-3).
FIGURE 2. Average contraction percentage of FPCL with treatment (MTX) and control.

FIGURE 3. The best average contraction percentage of FPCL with treatment (MTX, TA, combination of MTX and TA) and control.

FIGURE 1 and 2 show that all treatments with various doses of TA and MTX given separately caused a statistically significant (p<0.05) inhibition on FPCL contraction until day 2, compared to control. The inhibition of FPCL contraction was in dose-dependent manner, a higher dose of TA or MTX may paradoxically increase inhibition of FPCL contraction.

From the combination of TA and MTX groups (data were not shown), we found a similar result with treatment group which given separately. All various doses combination of TA and MTX caused a statistically significant (p<0.05) inhibition on FPCL contraction until day 2, compared to control. A higher dose of combination treatment might increase the inhibition of FPCL contraction. The best inhibition of FPCL contraction from each combination of TA and MTX was found in all various doses of MTX with 20 µM TA.

FIGURE 3 shows that all treatments could inhibit FPCL contraction until day 2 (p<0.05). The highest inhibition of FPCL was found in combination of TA 20 µM + MTX 7 µM (p<0.05). The treatment that could inhibit FPCL contraction until day 3 was only group MTX 3.5 µM + TA 20 µM combination (post-hoc test). This result indicated that a combination of TA and MTX would be stronger in inhibiting keloid fibroblast activities than TA and MTX given separately.

DISCUSSION

Experiments with cells derived from keloid tissue revealed a number of abnormalities in cellular functions, such as in proliferation, apoptosis, or expression of growth factors. The most important factor is TGF-α, followed by VEGF, IL6, and TNF-α. Keloid explants embedded in collagen lattice were morphologically similar with in vivo wound healing, it could be used for maintaining long-term viable keloid dermal explants until 6 weeks as a tool for investigating the pathogenesis of keloid scar formation. FPCL were used for investigating TGF-β, FGF, 5-FU, chitin, and chitosan on keloid.

This study (FIGURE 1-3) showed the differences of average contraction percentage of FPCL with treatment and control on day 1 until 3. This result indicated a viable fibroblast keloid and the consistency of FPCL contraction due to the treatment.

Triamcinolone acetonide (TA) could alter matrix extracellular due to fibroblast proliferation and TGF-β. Cruz and Korchin found that 10 µM TA caused statistically significant growth inhibition of keloid and fetus fibroblasts. Carrol found that 10 and 20 µM TA caused a statistically significant decrease in the level of TGF-β keloid fibroblast on day 1. Exposure with 5 µM TA did not cause a statistically significant decrease in the level of TGF-β. A higher dose of TA may increase cellular proliferation.

The result from our study (FIGURE 1) showed that not only 10 and 20 µM TA, but also 5 µM TA caused a statistically significant (p<0.05) inhibition of FPCL contraction until day 2, compared to control. The different results may be caused by different methods, previous study were not used.
FPCL and inhibition of FPCL were not only caused by TGF-β. The inhibition of FPCL contraction was in dose-dependent manner, a higher dose of TA or MTX might paradoxically increase inhibition of FPCL contraction.

Methotrexate (MTX) had been shown to suppress TNF-α due to apoptosis. Neurath found that 1-10 µg/ml MTX on murine caused suppression TNF-α and IL-6. Sigmundsdottir found that 1-10 µM MTX markedly reduced the expression of vascular E-selectin and ICAM 1 in psoriatic skin, and no further inhibition was observed after 10 µM MTX. This result was similar with our study. FIGURE 2 shows that 1.75, 3.5 and 7 µM MTX caused a statistically significant (p<0.05) inhibition on FPCL contraction until day 2, compared with control it was due to the inhibition of fibroblast proliferation, TNF-α, or may be the other factors, on dose-dependent.

The study of combination MTX with other chemotherapy or immnosuppressant for cancer or rheumatoid arthritis had been conducted. The target mechanism was VEGF, TGF B1 and TNF-a. Low-dose MTX with cyclophosphamide caused significant decreased level of VEGF in breast cancer, and with dexamethasone in rheumatoid arthritis. Combination of 5-FU and cyclophosphamide caused significant decreased plasma TGF-β1 concentration in breast cancer. Muzaffar and Tonkin reported a success in treating keloid with oral low dose MTX combined with reconstruction syndactily with no recurrences. In our study, all treatments with various doses of MTX and TA given separately or in combination could inhibit FPCL contraction until day 2 (p<0.05), except in group MTX 3.5 µM + TA 20 mM combination that could inhibit FPCL contraction until day 3 (p<0.05) (post-hoc test). That combination might be an ideal dose to get synergism effect. The highest inhibition of FPCL was found in combination of TA 20 µM + MTX 7 µM (p<0.05). This result indicated that combination of TA and MTX would inhibit further the keloid fibroblast activities than TA or MTX given separately. This result might be based on synergistic or additive effects of the drugs.

To summarize, we had demonstrated that the inhibition of FPCL contraction was dose-dependent, a higher dose of TA or MTX might paradoxically increase the inhibition of FPCL contraction. The combination of TA and MTX would inhibit further keloid fibroblast activities than TA or MTX given separately; strongly suggesting based on synergistic or additive effects of the drugs. These data suggested a new therapeutic approach for keloid.

REFERENCES


