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Association of fracture configuration and callus formation with a concentration of proinflammatory cytokines in children with long bone fractures

Povezanost tipa preloma i formiranja kalusa sa koncentracijom proinflamatornih citokina kod dece sa prelomima dugih kostiju

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Abstract

Background/Aim. The inflammatory response is of utmost importance in bone healing, but the precise role of cells and cytokines remains unclear. In our study, we examined the association between interleukin-1β (IL-1β), tumor necrosis factors alpha (TNF-α), monocyte chemoattractant protein-1 (MCP-1) and interleukin-8 (IL-8) concentrations, fracture configuration, and callus formation. **Methods.** Serum cytokine concentrations were determined in 78 non-obese children with long bone fractures (group 1), 10 children with finger fractures (group 2), and 10 healthy controls (group 3). Blood samples were taken immediately after fracture upon hospital admission for groups 1 and 2. Differences in cytokine concentrations were analyzed among groups and categorized according to fracture configuration and callus formation. **Results.** IL-1β and TNF-α levels were lower in patients that went on to produce incomplete callus compared with patients that formed complete callus. Surprisingly, the average IL-1β concentration was highest in the healthy control group. The only significant correlation between IL-1β and TNF-α was in the group with moderate callus formation. MCP-1 level was slightly increased in all patient groups compared to controls, with no mutual difference. An average IL-8 level showed a clear decrease tendency from the group with incompletely formed callus toward the group with completely formed callus compared to controls, without significant difference. Children with epiphysiolysis had the lowest concentrations of cytokines compared with all other fracture types including transverse, oblique, and spiral. There were significantly lower concentrations of IL-1β and MCP-1 in patients with less fragment displacement compared with patients with greater fragment displacement. **Conclusion.** The systemic inflammatory response is important in physiological bone healing. High early production of IL-1β, TNF-α, and MCP-1 is associated with greater callus formation and better healing outcome, while increased IL-8 level is associated with poor callus formation and worse healing outcome. Our results indicate that epiphysiolysis and larger fragment displacement are associated with delayed fracture healing.

Key words:
fractures, bone; fracture healing; child; adolescent; bony callus; interleukin-1beta; tumor necrosis factor-alpha; monocyte chemoattractant protein-1; interleukin-8; prognosis.

Apstrakt

Uvod/Cilj. Inflamatorni odgovor je od izuzetne važnosti u zarastanju koštanih preloma, iako je precizna uloga ćelija i citokina nejasna. U našoj studiji ispitivali smo povezanost vrednosti interleukina-1β (IL-1β), faktora nekroze tumora-alfa

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(TNF-α), monocitni hemoatraktantni protein-1 (MCP-1) i interleukina-8 (IL-8) sa konfiguracijom preloma i formiranjem kalusa. **Metode.** Serumska koncentracija citokina određivana je kod 78 negojazne dece sa prelomom dugih kostiju (grupa 1), 10 dece sa prelomom prstiju (grupa 2) i 10 zdrave dece (grupa 3 – kontrolna grupa). Uzorci krvi kod dece sa prelomima kostiju uzimani su odmah po prijemu u bolnicu (grupe 1 i 2). Razlike u koncentracijama citokina su analizirane između grupa i kategorisane prema konfiguraciji preloma i formiranju kalusa. **Rezultati.** Vrednosti IL1-β i TNF-α bile su niže kod bolesnika sa nedovoljno formiranim kalusom u odnosu na one sa kompletnim kalusom. Iznenađujuće, prosečna IL1-β koncentracija bila je najveća u kontrolnoj grupi. Jedina značajna korelacija između IL1-β i TNF-α bila je u grupi sa intermedijarno formiranim kalusom. MCP-1 je imao povišene vrednosti kod svih bolesnika u odnosu na kontrolnu grupu, bez međusobnih razlika. Prosečna vrednost IL-8 pokazala je jasan pad u grupi sa nekompletno formiranim kalusom u odnosu na grupu sa kompletno formiranim kalusom i kontrolnu grupu, ali bez značajne razlike. Deca sa epifiziolizom imala su najmanje koncentracije citokina u poređenju sa svim drugim tipovima preloma. Takođe smo detetkovali značajno niže koncentracije IL-1β i MCP-1 kod bolesnika sa manjim stepenom dislokacije u odnosu na veće dislokacije fragmenata. **Zaključak.** Sistemski inflamatorni odgovor je važan u fiziološkom zarastanju kostiju. Visoka rana produkcija IL1-β, TNF-α i MCP-1 je udružena sa boljim formiranjem kalusa i boljim zarastanjem kostiju, dok je povećana IL-8 koncentracija udružena sa lošim formiranjem kalusa i lošim zarastanjem kostiju. Naši rezultati su pokazali da su epifizioliza i veći stepen dislokacije fragmenata udruženi sa odloženim zarastanjem fraktura.

Ključne reči:
prelomi; prelomi, zarastanje; deca; adolescenti; kalus; interleukin-1beta; faktor nekroze tumora-alfa; monocitni hemoatraktantni protein-1; interleukin-8; prognoza.

Introduction

The most common injuries in children are fractures. In the past few decades, treatment has been improved, but still, some fractures heal slower and come with complications. For this issue, a deeper understanding of the bone healing process is of utmost importance.

One of the most astonishing processes in the human body is the healing of a fracture because the result of this process is not in scar formation but in forming a tissue similar to the preexisting. Fracture healing is a highly regulated process that consists of the following phases: the inflammatory phase, phase of reparation, and remodeling phase [1]. Although it is highly known that chronic production of inflammatory cytokines has a negative effect on bones, brief and precisely monitored production of proinflammatory cytokines is crucial for tissue regeneration [2]. The inflammatory phase is of utmost importance for the successful healing of the fracture because the proinflammatory cytokines released during this phase initiate further signaling pathways, which culminate with the healing of the above-mentioned fracture. For the first 24 hours, some proinflammatory cytokines like tumor necrosis factor-alpha (TNF-α) and interleukin-1β (IL1-β) are produced in the area of the injury [3, 4]. Values of the TNF-α and IL-1β achieve their maximum levels at the beginning of the fracture healing [4, 5]. The influx of the inflammatory cells at the place of the injury is affected by chemoattractive chemokines, where the most important factor is monocyte chemoattractant protein-1 (MCP1) which controls the movement of monocytes from the bone marrow to the blood flow and from there towards the place of inflammation [6]. Few hours after the fracture, the phase of reparation starts, and it is affected by local and systemic production of numerous growth and differentiation factors [7]. When the fracture is finally mended, the newly formed tissue must adapt to its function, and all this is happening during remodeling phase [1].

There are a few studies about the correlation of proinflammatory cytokines and fracture types, or fragment displacement, but it is proven that highly unstable fragments do slow down angiogenesis and make the newly formed bone tissue unable to fill the gaps [8].

All this leads us to further study the association between a few proinflammatory cytokines: IL-1β, TNF-α, MCP-1, and interleukin-8 (IL-8), and callus formation, also types of bone fractures and fragment displacement. Last but not least, we need a better understanding of these interactions so that we could further improve the methods of fracture healing.

Methods

All child patients were admitted, diagnosed, and treated at the Department of Orthopedics and Joints/Bone Trauma, Institute for Health Protection of Mother and Child ”Dr. Vukan Čupić”, Belgrade, Serbia. The parents of the tested children gave informed written consent for the participation of their children in the study. This study was approved by the Ethics Committee of this institution (No 8/26, 13/10/2015). The study included both boys and girls, age span from 4 to 18 years. There was one group of children with long bone fractures (group 1, n = 78), one group with fingers or small bone fractures (group 2, n = 10), and one control group of children that were admitted to the Clinic with a diagnosis of extremity trauma, but without proof of a fracture (group 3, n = 10). None of the children were obese [body mass index (BMI) 15–24 kg/m^2].

Children with other injuries apart from long bone fracture, children with systemic diseases of connective tissue, malignant, metabolic diseases, obese children (BMI over 24.0 kg/m^2), and children with congenital anomalies of the skeletal system were not involved in the study.

The study was a cross-sectional investigation. Sample of 2 mL venous blood was taken from the cubital vein in all children 1 hour after admission. After the serum separation, the samples were frozen at -70°C until testing. The concentrations of cytokines were determined with a

commercial flow cytometric test (LEGENDplex 13-plex Human Adipokine Panel) on a flow cytometer Beckman Coulter FC500. The concentrations of IL1-β, TNF-α, MCP-1, and IL-8 in all patients were measured.

All patients had anteroposterior (AP) and lateral radiographs taken at 5-time points: immediately, preoperatively, postoperatively, 7 days postoperatively, 21 days postoperatively, and after removing the plaster cast. The type of fracture was classified as epiphysiolysis, transverse, oblique, or spiral. The fragment displacement was classified as undisplaced, displaced < 1 cm, and displaced > 1 cm. Callus formation was classified according to radiological analysis as incomplete (< 25%), partial (< 50%), and complete (> 75%). Patient files were written about their earlier fractures, infections of the upper respiratory tract, allergies, and eating habits.

We used parameters of descriptive statistics to analyze group variability and to estimate the central tendency of data. Analysis among more than two groups, groups according to fracture type (epiphysiolysis, transverse, oblique, spiral), fragment displacement (without displacement, displacement < 1 cm, displacement > 1 cm), and among groups depending on the degree of callus formation (< 25%, < 50%, > 75%) were performed using one-way analysis of variance (ANOVA), with Bonferroni post-testing. The Mann-Whitney test was used for all other comparisons between two independent groups. All statistical analyses were done using the statistical package GraphPad Prism 5.01 (GraphPad Prism Software Inc. California, USA).

Results

The results are shown in Tables 1 and 2.

Cytokines and callus formation

The average IL-1β concentration was lower in the groups 1 and 2 compared to the group 3. There was a significant difference between patients that had complete callus and those in the group 2. Although IL-1β concentration was lower in patients with insufficient callus compared with patients with completely formed callus, these differences were not statistically significant. On the other hand, the group 1 had almost twice the TNF-α concentrations of the groups 2 and 3. Patients with complete callus had the highest average TNF-α concentration, significantly greater than the patients in the groups 2 and 3.

Because of simultaneous increases in both IL-1β and TNF-a, a correlation analysis was performed. The only significant correlation was in the patients with incomplete callus formation.

MCP-1 and IL-8 levels were also higher in the group 1 than in the groups 2 and 3, but the differences were not significant. Patients with incompletely formed callus had the highest IL-8 concentrations.

Cytokines and fracture configuration

There were significant differences in IL-1β concentrations in children with oblique, transverse, and spiral long bone fractures when compared with patients in the group 2 (Table 1) and when comparing the groups 2 and 3 (Table 2). Children with epiphysiolysis had lower IL-1β values comparing with children with spiral long bone fractures.

TNF-α concentration was significantly higher in children with transverse long bone fracture compared with those in the groups 2 and 3.

Table 1

Average concentrations of IL1-β, TNF-α, MCP-1, and IL-8 in blood samples of children with bone fractures

Groups	n	Cytokines (pg/mL), mean ± standard deviation			
		IL-1β	TNF-α	MCP-1	IL-8
Group 1 (long bone fractures)					
callus formation					
incomplete	6	46 ± 10	97 ± 54	98 ± 18	2,823 ± 1,117
intermediary	53	53 ± 18	82 ± 50	98 ± 51	2,351 ± 1,027
complete	19	60 ± 19 [a]	114 ± 63 [a, b]	101 ± 27	2,141 ± 437
fracture type					
epiphysiolysis	8	36 ± 21 [b]	47 ± 21	57 ± 19 [a,b,c]	1,807 ± 446
oblique	20	54 ± 16 [c]	87 ± 70	99 ± 36 [d]	2,476 ± 970 [a]
transverse	39	52 ± 18 [e]	104 ± 63 [c, d]	94 ± 42 [e]	2,404 ± 1038
spiral	11	72 ± 25 [b,d,f]	57 ± 38	148 ± 60 [c,d,e,f,g]	2,038 ± 680
dislocation					
no	8	63 ± 18 [h,]	99 ± 20 [e]	97 ± 31	2,955 ± 1,028 [b,c,d]
< 1 cm	62	50 ± 19 [i, j]	92 ± 64 [f]	91 ± 35 [h]	2,369 ± 966 [e]
> 1 cm	8	70 ± 20 [i, k]	68 ± 72	158 ± 68 [h, i, j]	1,666 ± 666 [b]
Group 2 (finger fractures)	10	38 ± 13 [h, j, k, l]	48 ± 30	74 ± 24 [i]	1,915 ± 446 [c]
Group 3 (controls)	10	65 ± 22 [l]	44 ± 29 [e,f]	88 ± 39 [j]	1,784 ± 348 [d, e]

Superscripted letter depicts significant difference between specific groups (precise data are given in Table 2).
IL-1β – interleukin-1β; TNF-α –tumor necrosis factor-α; MCP-1 – monocyte chemoattractant protein-1; IL-8 – interleukin-8.

Table 2

Significant differences in cytokine concentrations among analyzed groups of patients according to degree of callus formation, fracture type, and fragment dislocation

Cytokine	Marker	Group	vs.	Group	*p*
IL-1β					
callus	a	complete	/	control finger fractures	0.0450
fracture	b	epyphysiolysis	/	spiral	0.0485
	c	oblique	/	control finger fractures	0.0213
	d	transverse	/	spiral	0.0191
	e	transverse	/	control finger fractures	0.0258
	f	spiral	/	control finger fractures	0.0070
	g	control	/	control finger fractures	0.0104
dislocation	h	no	/	control finger fractures	0.0208
	i	< 1 cm	/	> 1 cm	0.0343
	j	< 1 cm	/	control finger fractures	0.0346
	k	> 1 cm	/	control finger fractures	0.0109
	l	control finger fractures	/	control	0.0104
TNF-α					
callus	a	complete	/	control finger fractures	0.0131
	b	complete	/	control	0.0177
fracture	c	transverse	/	control finger fractures	0.0040
	d	transverse	/	control	0.0014
dislocation	e	no	/	control	0.0012
	f	< 1 cm	/	control	0.0095
MCP-1					
fracture	a	epyphysiolysis	/	oblique	0.0369
	b	epyphysiolysis	/	transverse	0.0433
	c	epyphysiolysis	/	spiral	0.0167
	d	oblique	/	spiral	0.0291
	e	transverse	/	spiral	0.0024
	f	spiral	/	control finger fractures	0.0022
	g	spiral	/	control	0.0140
dislocation	h	< 1 cm	/	> 1 cm	0.0066
	i	> 1 cm	/	control finger fractures	0.0016
	j	> 1 cm	/	control	0.0025
IL-8					
fracture	a	oblique	/	control	0.0312
dislocation	b	no	/	> 1 cm	0.0186
	c	no	/	control finger fractures	0.0207
	d	no	/	control	0.0045
	e	< 1 cm	/	control	0.0441
	b	no	/	> 1 cm	0.0186

Superscripted letter depicts significant difference between specific groups, as given in Table 1 (Mann-Whitney test). IL-1β – interleukin-1β; TNF-α – tumor necrosis factor-α; MCP-1 – chemoattractant protein-1.

Significantly lower values of MCP-1 were found in children with epiphysiolysis than with other fracture configurations. Children with spiral long bone fractures had significantly higher MCP-1 values than with any other fracture configuration or groups.

Significant differences in IL-8 concentrations were only seen between children with oblique long bone fractures and controls.

Cytokines and bone fragment displacement

All patients in the groups 1 and 3 had significantly higher IL-1β concentrations compared with children in the group 2. In children with displacement < 1 cm, the concentrations of IL-1β were significantly lower than in children with greater displacement.

Patients without fragment displacement and with displacement < 1 cm had significantly higher TNF-α concentrations compared with patients in the group 3. The highest concentration of IL-8 was seen in patients without fragment displacement, and it was significantly higher than in patients from the groups 2 and 3 and patients with displacement > 1 cm.

Children with displacement < 1 cm, as well as those in the groups 2 and 3 had significantly lower MCP-1 concentrations compared with children with displacement > 1 cm.

Discussion

Even with countless studies, the process of fracture healing remains mostly unexplored. It goes on in 3 different but overlapping phases: the early inflammatory phase, phase of regeneration, and remodeling phase [9]. The use of anti-inflammatory drugs during the early phase of fracture healing in many studies has shown that the healing process

was considerably worse and slower. This implies that the inflammatory phase could be crucial for the successful healing of the fracture [10–12]. Proinflammatory cytokines are known for their destructive and catabolic effect on bones, but these same proinflammatory cytokines are making the fracture healing faster [13]. Differing from the uncontrolled inflammation that we see in bone pathology, inflammation in fracture healing of a healthy subject is short and highly regulated [1]. Many studies have shown that the precise regulation of the initial inflammatory phase is one of the determining factors for fracture healing and that the proinflammatory cytokines, like TNF-α and IL-1β, have a major role in initiating the repair process [2, 14, 15].

TNF-α shows maximal values in the first 24 hours after the fracture. After that, it shows decreasing levels during the cartilage formation. Then the values grow again during the remodeling phase [14, 15]. This is in accordance with our results, where all the patient groups upon admission had almost doubled TNF-α concentration compared to the healthy controls. Studies conducted on mice show that the absence of TNF-α slows the healing of a fracture for at least a couple of weeks. Contrary to that, TNF-α deficient mice have a normal skeleton which implies that TNF-α has a specific role in postnatal fracture healing [15]. These findings are also consistent with our results where patients from the group 2 with completely formed callus had the highest average TNF-α concentration, significantly increased compared to both control groups, and values of this cytokine were rising among patient groups from the group with minimal callus formation, where they were the lowest, towards the group with completely callus formation, where they were the highest. Macrophages are the main source of TNF-α and IL-1β during the inflammatory phase [3, 4]. Whilst they use different signal pathways, effects of IL1-β on the bone mostly overlap with effects of TNF-α [16], which was also shown in our study where a similar increase in TNF-alpha and IL-1β was seen from patients with insufficient callus to the group with completely formed callus. It is of utmost importance to specify that a balanced immune response is crucial for a successful fracture healing [17, 18] because the scarcity of TNF-α delays the fracture healing, whilst abundant production of TNF-α damages the bone [19, 20].

The conclusion is that precise regulation of signaling pathways is necessary in each of the healing phases in order to maintain the balanced concentration of different cytokines during the different phases of fracture healing. We are underlying this fact because it could be the explanation for the fact that patients from the group with minimal callus formation had the highest IL-8 concentrations and that the average IL-8 level showed a clear decrease tendency toward the group with completely formed callus. It could indicate that the high and long production of IL-8, which is a neutrophil chemoattractant factor, could lead to a high and uncontrolled neutrophil infiltration. It is widely known that excessive neutrophil degranulation is common in many inflammatory diseases, and as such, it can cause tissue damage as well as the delay of the healing process [21].

The influx of inflammatory cells to the place of the injury is also affected by chemoattractive chemokines. CCR2/CCL2 (MCP-1) controls the movement of the monocytes after the injury from bone marrow to the blood flow and from there to the place of inflammation [6]. Studies conducted on mice that were missing the CCR2 gene have shown that influx of macrophages to the place of the injury, in this case, is considerably smaller in comparison with the wild mice [22, 23]. Another study on mice with deficiency of CCR2 gene was comparing the size and thickness of the callus between these mice and the wild ones in different time spans after the injury, and it has shown that the healing of CCR2 deficient mice was delayed in comparison to the wild type [24]. The results of this study prove the importance of CCR2/CCL2 interaction for the formation of callus and are in accordance with our results where the patients with the lowest callus formation had the lowest concentration of CCL2 while this value was rising upon patients with completely formed callus.

There are not many studies about the correlation between the type of bone fractures and cytokines concentration with which we could compare our results, but the most obvious thing from our results is that the lowest concentrations of all investigated cytokines were observed in children with epiphysiolysis compared with all other types of fractures. One of the recently conducted studies has shown the importance of the formation of the initial hematoma as the first influx of the inflammatory cells, which will produce cytokines and be able to initiate further influx of immune cells to the place of the injury [25]. On the other hand, countless studies conducted to this day have shown that one of the major complications of epiphysiolysis is the insufficient blood supply of the femoral head due to the damage of epiphyseal blood vessels. Damage of epiphyseal blood vessels can be the consequence of the fracture or the result of high capsule pressure due to internal intracapsular bleeding [26, 27]. This insufficient blood supply could lead to poor formation of the initial hematoma and inadequate influx of inflammatory cells, which explains the lowest cytokine concentrations in children with epiphysiolysis in our study. Moreover, according to our previously discussed results about the influence of cytokine concentrations on callus formation and bone healing, it is probable that this poor influx of inflammatory cells leads to delayed healing of the fracture in the cases of children with epiphysiolysis.

We were also analyzing the association between bone fragment displacement and proinflammatory cytokine concentrations. Our study showed that there were significantly lower concentrations of proinflammatory cytokine IL-1 beta and MCP-1 in patients with less fragment displacement (displacement less than 1 cm) compared with patients with larger fragment displacement (displacement bigger than 1 cm). A study that has been recently conducted could explain these findings. This study was conducted on sheep, where the removal of the initially formed hematoma and its effect on fracture healing was tested [28, 29]. In this study, it has been shown that the removal of the initially formed hematoma leads to a new influx of inflammatory cells. This differs from the physiological process of healing, where the anti-inflammatory signal grows stronger for 24 hours after the fracture and thus considerably reduces the inflammation process [18]. This new inflammatory

impulse could further delay the inflammatory phase. If we compare these findings with our results, we could assume that a bigger fragment displacement produces more mechanical instability, which can damage the primarily formed hematoma and set a new inflammatory impulse. Additionally, this could be the reason for higher cytokine concentrations in patients with bigger fragment displacement. We must not forget that only a highly regulated inflammatory process can further improve fracture healing and, *vice versa*, every extension of the inflammatory phase leads to slower fracture healing [18, 27, 30].

Conclusion

The systemic inflammatory response is important in physiological bone healing. High early production of IL-1β, TNF-α, and MCP-1 is associated with greater callus formation and better healing outcome, while a high level of neutrophil chemotactic cytokine (IL-8) is associated with poor callus formation and worse healing outcome. Based on our results, there is delayed healing fracture in patients with epiphysiolysis and bigger fragment displacement.

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