Concise Report

Calcinosis in juvenile dermatomyositis: a possible role for the vitamin K-dependent protein matrix Gla protein

M. J. H. van Summeren[1], W. G. M. Spliet[2], A. van Royen-Kerkhof[1], C. Vermeer[3], M. Lilien[4], W. Kuis[1] and L. J. Schurgers[3]

Objectives. The aims of the present study were to investigate whether the calcification inhibitor matrix Gla protein (MGP) is expressed in muscle biopsies of patients with juvenile dermatomyositis (JDM), and whether different forms of MGP are differentially expressed in JDM patients with and without subcutaneous calcifications.

Methods. Muscle tissue from six JDM patients (three without calcinosis, two with calcinosis and one recently diagnosed patient), four patients with muscular dystrophy, three patients with IBM and five normal histological control subjects was used for immunohistochemistry staining using novel antibodies to different conformations of MGP.

Results. In the JDM patients, all forms of MGP [non-carboxylated MGP (ucMGP), carboxylated MGP (cMGP), non-phosphorylated MGP (serMGP) and phosphorylated MGP (pserMGP)] were more intensely stained in the perifascicular compared with the central muscle fibres. In addition, these MGP species were demonstrated in the pathological muscle fibres of IBM and dystrophy patients, but hardly in normal histological muscle tissue. In JDM patients with calcifications, only pserMGP was increased compared with those without calcifications. All forms of MGP were also found in various staining intensities in the microvasculature and macrophages of normal histological and disease biopsies.

Conclusions. MGP was expressed at the site of muscle damage in JDM patients as well as in patients with muscular dystrophy and IBM. The difference in staining intensity of pserMGP appeared to distinguish between JDM patients with and without calcifications, whereas cMGP, the other functional form, was equally expressed.

KEY WORDS: Vitamin K-dependent proteins, Matrix Gla protein, Juvenile dermatomyositis, Immunohistochemistry, Muscle.

Introduction

Patients with juvenile dermatomyositis (JDM) may develop subcutaneous calcification in the course of the disease [1]. The pathophysiology of calcinosis in JDM is still largely unknown [2, 3]. A remarkably increased urinary γ-carboxyglutamic acid (Gla) output was found in JDM patients, especially those with calcifications [4]. Furthermore, Gla-containing proteins have been demonstrated in several types of pathological calcifications including calcium-containing material extruded from skin and subcutaneous plaques from patients with dermatomyositis [5].

Matrix Gla protein (MGP) is a vitamin K-dependent protein which contains five Gla residues. In recent years, the role of MGP as an inhibitor of calcification in vascular and cartilage calcification has become overt [6]. Vitamin K serves as a cofactor in the post-translational γ-carboxylation of MGP. In this process, the conversion of selective protein-bound glutamate (Glu) residues into Gla takes place, which is essential for protein function [7]. Besides its five Gla residues, mature MGP contains three serine residues that may be phosphorylated. Several conformation-specific antibodies of MGP for immunohistochemistry have been developed [8].

We hypothesized that MGP may be involved in the pathogenesis of calcinosis in JDM and investigated the localization of the different forms of MGP in muscle biopsies from JDM patients with and without calcification.

Methods

Patient material

For tissue samples, we had access to a database for human tissue collected and registered by the Department of Pathology of the University Medical Center Utrecht. Specimens were collected and classified according to histological examination. Since muscle biopsies are not obligatory to confirm the diagnosis of JDM we obtained muscle tissue from six JDM patients (2 males, 4 females; average age 10.5 yrs) only (period 2001–07). All but two biopsies were taken at time of the onset of disease. Two of six JDM patients had developed severe multilocalized calcifications during the disease course, starting 1.5 and 2 yrs after disease onset. The patients with calcifications had a biopsy at the time when calcifications had already developed. No biopsies were taken from the areas of calcinosis due to concerns about the risk of poor healing. The average clinical follow-up time of JDM patients after biopsy was 4.0 yrs (range 0.1–6 yrs). Since the biopsy of one patient was recently taken, it is uncertain whether calcinosis will develop in this patient.

As negative control material, muscle biopsies from five patients with myopathic complaints, but normal histology and without definitive clinical diagnosis, were used. These samples showed no muscle fibre abnormalities or signs of inflammation. Muscle biopsies from patients with confirmed muscle disease were used as disease controls; three patients had IBM and four patients had muscular dystrophy.

The Department of Pathology has consent from the Medical Ethics Committee of the University Medical Centre Utrecht to use diagnostic patient material for research purposes unless the patient gives an explicit refusal as is stated in patient information brochures.

MGP antibodies

Monoclonal antibodies against the different MGP conformations used for immunohistochemistry were provided by VitaK BV

[1]Department of Pediatric Immunology, [2]Department of Pathology, University Medical Centre Utrecht, Utrecht, [3]Department of Biochemistry, University of Maastricht, Maastricht and [4]Department of Pediatric Nephrology, University Medical Centre Utrecht, The Netherlands.

TABLE 1. Quantitative MGP immunostaining in JDM patients and control patients

Biopsy	Histology	Pathological muscle fibres[a]				Normal muscle fibres[a]				Macrophages				Microvasculature			
		uc	c	ser	pser	uc	c	ser	pser	uc	c	ser	pser	uc	c	ser	pser
1	Normal	0	0	0	+	0	0	0	+	+	+	++	++	+	+	0	++
2	Normal	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
3	Normal	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
4	Normal	0	0	0	0	0	0	0	0	++	++	++	++	++	++	++	++
5	Normal	0	0	++	+	0	0	0	+	++	++	++	++	++	++	++	++
6	JDM+	+	+	0	++	0	0	0	++	++	++	0	++	++	++	0	++
7	JDM+	++	+++	0	+++	0	0	0	++	++	++	0	++	++	++	0	++
8	JDM−	0	+	0	+	0	0	0	+	+	++	0	+	+	++	0	+
9	JDM−	++	+	0	+	0	0	0	+	++	++	0	+	++	+	0	+
10	JDM−	++	+	0	+	0	0	0	+	++	++	0	+	++	+	0	+
11	JDM?[b]	++	++	+++	+++	0	+	++	+	++	++	++	++	++	++	+	++
12	Dystrophy	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
13	Dystrophy	0	+	+	+	0	0	+	0	++	++	++	++	++	++	+	++
14	Dystrophy	+	++	++	++	0	0	0	0	++	++	++	++	++	++	++	++
15	Dystrophy	+	+	+	++	0	0	0	+	++	++	++	++	++	++	++	++
16	IBM	+	++	++	+	0	0	0	0	++	++	++	++	++	++	++	++
17	IBM	+	+	++	+	0	0	0	0	++	++	++	++	++	++	+	++
18	IBM	+	+	++	++	0	0	0	+	++	++	++	++	++	++	++	++

The table shows the staining intensity of ucMGP, cMGP, serMGP and pserMGP in muscle biopsies of JDM patients with (JDM+) and without (JDM−) calcification, IBM patients (IBM), patients with muscular dystrophy (dystrophy) and negative control patients (normal). The staining intensity of pathological and normal muscle fibres, macrophages and microvasculature was scored from 0 (no staining), + (low), ++ (medium) to +++ (intense). [a]In biopsies of JDM patients and negative control patients, Pathological muscle fibres refer to the perifascicular muscle fibres whereas Normal muscle fibres refer to the central muscle fibres. [b]This biopsy was recently taken and presently it is uncertain whether calcinosis will develop in this patient.

(Maastricht, The Netherlands). In brief, monoclonal antibodies (mAb) were raised against the non-carboxylated human MGP Gla-domain (residues 35–49; designated as mAb-ucMGP), the carboxylated human MGP Gla-domain (residues 35–54; designated as mAb-cMGP), the non-phosphorylated human MGP (residues 3–15; designated as mAb-serMGP) and phosphorylated human MGP (residues 3–15; designated as mAb-pserMGP). The monoclonal antibodies were selected for their specificity against ucMGP, cMGP, serMGP and pserMGP, respectively. The method to obtain the antibodies for ucMGP, cMGP and serMGP has been described before [8]. The conformation-specific antibody for pserMGP has not yet been described but was raised according to the method as described by Schurgers et al. [8].

Immunohistochemistry of ucMGP, cMGP, serMGP and pserMGP

All tissues were stained for haematoxylin and eosin (HE) to demonstrate tissue integrity. For immunostaining with any of the four mAb MGP antibodies, sections were incubated with anti-ucMGP (0.9 µg/ml), anti-cMGP (1.0 µg/ml), anti-serMGP (1.0 µg/ml) or anti-pserMGP (0.75 µg/ml). All antibodies were diluted in blocking reagent (Roche Diagnostics, Mannheim, Germany). Biotinylated sheep anti-mouse IgG (Amersham Biosciences, Little Chalfont, UK) was used as a second antibody, followed by incubation with avidin-linked alkaline phosphatase complex (Dako, Glostrup, Denmark); staining was performed by the alkaline phosphatase kit I (Vector Laboratories, Burlingame, CA, USA). All specimens were counterstained using haematoxylin and sections were mounted using Imsol mount.

Qualitative and semi-quantitative analysis of MGP staining

The overall pattern of immunostaining and the staining intensity was examined microscopically in the microvasculature, fibroblasts, macrophages and muscle fibres of the biopsies by an experienced pathologist. The staining intensity was scored from 0 (no staining), + (low), ++ (medium) or +++ (intense). Because the typical muscular abnormalities of JDM patients are localized in the perifascicular muscle fibres, a distinction between central and perifascicular muscle fibres was made when comparing the biopsies of the JDM patients with the normal histological control tissue. When comparing the biopsies of the JDM patients with the disease controls, a distinction between pathological and normal muscle fibres was made.

Results

The immunostaining results for the different MGP antibodies in JDM patients with and without calcifications, control patients with normal muscle histology, IBM patients and muscular dystrophy patients are shown in Table 1. Figure 1 shows the immunohistochemical localization of MGP species in muscle biopsies of a control patient and JDM patient with subcutaneous calcifications. In the control biopsies, no staining of cMGP and ucMGP was found anywhere in muscle tissue. In two control patients only, pserMGP and serMGP were faintly stained in perifascicular and central muscle fibres.

In all JDM patients, MGP was mainly localized within degenerated atrophied (perifascicular) muscle fibres. The immunostained product of cMGP and pserMGP was seen in perifascicular fibres in all JDM patients. UcMGP was present in perifascicular muscle fibres in most JDM patients. PserMGP was present in central muscle fibres of all JDM patients. The staining pattern in the JDM patient who was recently diagnosed was quite different from the other JDM patients. This biopsy showed the presence of perifascicular-localized serMGP together with cMGP and serMGP in central muscle fibres. In JDM patients, all forms of MGP showed increased staining intensity in perifascicular muscle fibres in comparison with the negative control biopsies. The staining of all types of MGP was not clearly co-localized or up-regulated within inflammatory infiltrates (Fig. 1). When comparing the JDM patients with and without calcifications, an increased staining intensity of pserMGP was noted in the JDM patients with calcifications in perifascicular and central muscle fibres. In one JDM patient with calcinosis, a higher staining intensity for cMGP in the damaged muscle fibres was demonstrated in comparison with the JDM patients without calcinosis.

In most disease control biopsies, all types of MGP were found in the abnormal muscle fibres. In normal muscle fibres of these biopsies, no cMGP or ucMGP was found. In three patients only, pserMGP and serMGP were weakly stained in normal muscle fibres.

In the majority of biopsies (of JDM, disease control and negative control patients), all species of MGP were found in macrophages and microvasculature, although subtle differences
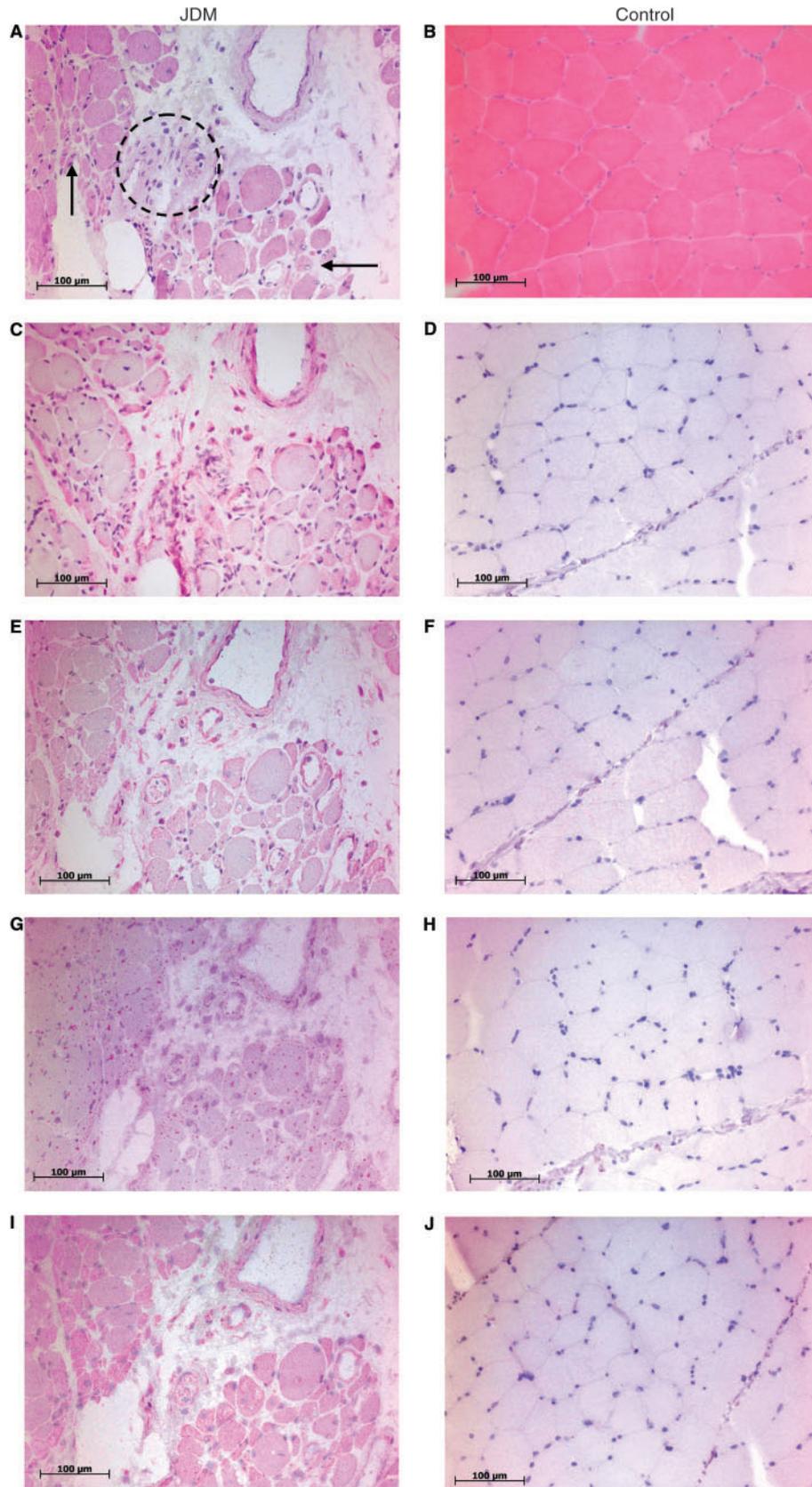
FIG. 1. Immunohistochemical localization of MGP species in muscle biopsies of a JDM patient (with calcinosis) and a negative control patient. **A** and **B** represent HE staining in a JDM patient and negative control patient, respectively. **A** shows the typical perifascicular atrophy of muscle cells (indicated by the arrows) as well as a small inflammatory infiltrate in the epimysial connective tissue (indicated by the circle) as can be found in JDM biopsies. **B** shows normal muscle tissue. **C** and **D** represent ucMGP staining in a JDM patient and negative-control patient, respectively. **E** and **F** represent cMGP staining in a JDM patient and negative control patient, respectively. **G** and **H** represent serMGP staining in a JDM patient and negative control patient, respectively. **I** and **J** represent pserMGP staining in a JDM patient and negative control patient, respectively. In the biopsy of the JDM patient, staining of different species of MGP is demonstrated (in pink/red colour) in perifascicular muscle cells whereas in the negative control muscle tissue, no MGP is demonstrated.

in staining intensities within and between patient groups were noted.

Discussion

Our study describes the identification of the calcification inhibitor MGP at the site of muscle damage in JDM patients with and without calcifications using novel conformation-specific MGP antibodies. In addition, MGP was found in the microvasculature and macrophages of normal and disease biopsies. Others have also reported the presence of mRNA and protein for MGP in macrophages and the arterial medial wall [8, 9] whereas the presence of MGP in striated muscle tissue was not demonstrated before. The possible role of MGP in (dermal) calcinosis was studied previously in another auto-inflammatory disease [10]. This study in scleroderma patients demonstrated the presence of MGP in skin biopsies, using only the serMGP antibody.

In JDM patients, chronic inflammation is presumably a necessary condition for the formation of dystrophic calcification [3]. The destruction of muscle fibres in JDM results from the deposition of complement in endomysial capillaries, which leads to swelling of endothelial cells and capillary necrosis and ultimately to perivascular inflammation and local ischaemia [11]. The resulting tissue injury is a trigger for the calcification process. Increased local calcium concentration, resulting from cell damage, leads to enhanced MGP expression [12]. A proposed mechanism of MGP to prevent (progression of) calcinosis is to bind tightly and selectively to calcium both in solution and in calcium crystal nuclei in order to prevent their growth and ability to seed daughter crystals [13]. In ectopic calcifications of JDM patients, hydroxyapatite was found to be present, indicating that osteoblast-like cells may also be involved [14]. Besides direct binding to calcium, Gla-containing MGP (cMGP) also inhibits cell differentiation into an osteoblastic phenotype, induced by BMP-2 [7]. Because the binding to BMP-2 is dependent on the presence of Gla-residues in MGP, insufficient levels of cMGP may predispose to calcification. Inadequate (local) vitamin K reserves or supply may lead to impaired carboxylation of MGP [15]. The question is thus raised whether supplementation with vitamin K in patients with JDM will increase the functionality of MGP and thereby may positively influence the development of calcinosis.

As only cMGP is presumed to fully exert its role as inhibitor of calcification, we had expected that the level of cMGP expression would be differentiating between JDM patients with and without calcifications. Only in one case of a JDM patient with calcinosis, a higher staining intensity for cMGP in the damaged muscle fibres was noted in comparison with the JDM patients without calcinosis. This observation suggests that cMGP is up-regulated but not in sufficient amount that calcification is prevented. Furthermore, the fact that the biopsies of the JDM patients with calcinosis were not taken at the site of the calcification may explain the similar expression patterns of cMGP. Given the focality of the calcification process, it may be that altered levels of cMGP can only be found at the site of the calcifications in the JDM patients. Surprisingly, the intensity of pserMGP staining appeared to distinguish between JDM patients with and without calcifications. Of course, this observation needs to be confirmed in larger numbers of patients. Besides the presence of Gla-residues, the activity of MGP may also be determined by the presence of phosphorylated serines [16]. The precise function of the phosphoserines in MGP is presently unknown. The extent of serine phosphorylation is probably regulated by a Golgi-associated protein kinase that can rapidly react to changes in the extracellular environment [17]. It remains unclear whether both post-translational modification processes (γ-carboxylation and phosphorylation) are simultaneously required for MGP to fully exert its function.

In the present study, MGP was also expressed in pathological muscle tissue of the other myopathies studied. Since the occurrence of calcification in IBM and dystrophy is not common, the expression of MGP at the site of muscle damage most likely represents a general protective mechanism. When the protective action of MGP fails due to insufficient expression or decreased functionality, this may contribute to the development of calcifications. The defensive mechanism of MGP is probably not disease-specific but related to the extent of inflammation and concomitant tissue damage. In JDM, muscular destruction, presenting as perifascicular atrophy, is more prominent compared with IBM [18]. The extensive inflammatory responses and tissue damage are likely to predispose JDM patients to calcinosis. Accordingly, clinical studies have shown that calcinosis in JDM patients is related to severity of disease and delays in diagnosis or delayed initial treatment [19].

To conclude, the expression of MGP in damaged muscle tissue probably represents a general protective mechanism against unwanted calcification that may fall short in some patients with JDM. Local availability of functional cMGP may be increased by supplementation with vitamin K. Additional studies are warranted to learn more about regulating factors in MGP serine phosphorylation, another potential determinant of MGP functionality.

Rheumatology key messages

- The calcification inhibitor MGP is expressed at the site of muscle damage in patients with juvenile dermatomyositis.
- The expression of MGP in these patients probably represents a general protective mechanism against harmful calcification.

Acknowledgements

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