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Fatty acid composition in matrix vesicles and in microvilli from femurs of chicken embryos revealed selective recruitment of fatty acids

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ABSTRACT

Hypertrophic chondrocytes participate in matrix mineralization by releasing matrix vesicles (MVs). These MVs, by accumulating Ca^{2+} and phosphate initiate the formation of hydroxyapatite. To determine the types of lipids essential for mineralization, we analyzed fatty acids (FAs) in MVs, microvilli and in membrane fractions of chondrocytes isolated from femurs of chicken embryos. The FA composition in the MVs was almost identical to that in microvilli, indicating that the MVs originated from microvilli. These fractions contained more monounsaturated FAs especially oleic acid than in membrane homogenates of chondrocytes. They were enriched in 5,8,11-eicosatrienoic acid (20:3*n*−9), in eicosadienoic acid (20:2*n*−6), and in arachidonic acid (20:4*n*−6). In contrast, membrane homogenates from chondrocytes were enriched in 20:1*n*−9, 18:3*n*−3, 22:5*n*−3 and 22:5*n*−6. Due to their relatively high content in MVs and to their selective recruitment within microvilli from where MV originate, we concluded that 20:2*n*−6 and 20:3*n*−9 (pooled values), 18:1*n*−9 and 20:4*n*−6 are essential for the biogenesis of MVs and for bone mineralization.

1. Introduction

Experimental evidence suggests that lipids are involved in the skeletal development and in bone formation. For example, phospholipids facilitate cartilage mineralization in the growth plate [1]. Findings from animal models indicated that a low dietary intake of *n*−6 polyunsaturated fatty acids (PUFAs) and increasing intake of *n*−3 PUFAs, especially long chain *n*−3 PUFAs, are beneficial for achieving higher bone mineral content, density and stronger bones [2,3]. Bone is a multifunctional organ that consists of a structural framework of mineralized matrix harboring a heterogeneous population of chondrocytes, osteoblasts, osteocytes, osteoclasts, endothelial cells, monocytes, macrophages, lymphocytes, and hematopoietic cells [4]. Mineralization competent cells, *e.g.*, osteoblasts and hypertrophic chondrocytes, which are present respectively in bones and in cartilages, release vesicular structures serving as an initial site for mineral formation. These vesicles called matrix vesicles (MVs), are derived from the cellular plasma membrane [5]. Lipids are integral components of MV membrane and provide a barrier to confine the contents of the vesicles. Once released into the extracellular matrix, these MVs accumulate Ca^{2+} and inorganic phosphate which finally leads to hydroxyapatite formation on the surface of the inner leaflet of the MV membrane. This process requires a dynamic and tightly regulated system to maintain ion homeostasis and cooperation between extracellular macromolecules and MV proteins [6,7]. The cell surface microvilli are the precursors of MVs. A retraction of supporting microfilament network under the plasma membrane is essential for the release of MVs from osteoblast [8] and chondrocyte [9]. The mechanism of MV biogenesis is not fully understood. The nature of fatty acids (FAs) is a key factor for the MV formation. The ability and degree of FAs to modulate the membrane organization are dependent of their sizes and morphologies. Peress et al. [10] first reported the lipid composition of the MV fraction isolated from

Abbreviations: MVs, matrix vesicles; PUFAs, polyunsaturated fatty acids; FAs, fatty acids; TNAP, tissue non-specific alkaline phosphatase.

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epiphyseal cartilage of fetal calves. The phospholipid composition of the MV and microvilli fractions was markedly different from that of the chondrocytes. These MVs and microvilli produced by epiphyseal cartilage cells [10,11] or by hypertrophic chondrocytes were enriched in cholesterol, phosphatidylserine, and sphingomyelin as in the case of MVs obtained from Saos-2 cell cultures [8]. However, the nature of FAs which are essential in the MV biogenesis was not determined. In this work we determined and compared the FA composition of MVs, microvilli and chondrocytes isolated from femurs of chicken embryos. The lipids were extracted and analyzed for their FA composition by thin-layer and gas–liquid chromatography.

2. Materials and methods

2.1. Isolation of MVs and microvilli fractions

MVs and microvilli were isolated from 17-days-old chicken embryos leg bones as reported [12]. The microvilli were prepared according to Jimenez et al. [13] with slight modifications (Fig. 1). The microvilli isolation was based on the property of $MgCl_2$ to precipitate membranous fractions except microvilli [14]. It was suggested that aggregation of membranous components occurs when bivalent cations establish cross-links between membranes. Microvilli do not aggregate because cross-linking by Mg^{2+} is established between contiguous anionic sites on the same microvillus. Leg bones from 40 animals *per* preparation (approximately 12 g of wet tissue) were cut into 1–3 mm thick slices and extensively washed in the ice cold synthetic cartilage lymph (SCL) medium [15] containing 1.83 mM $NaHCO_2$, 12.7 mM KCl, 0.57 mM $MgCl_2$, 100 mM NaCl, 0.57 mM Na_2SO_4, 5.55 mM glucose, 63.5 mM sucrose, and 16.5 mM 2-([2-hydroxy-1,1-bis(hydroxymethyl) ethyl]amino)-propanesulfonic acid (TES), pH 7.4. Bone slices were then digested with type-I collagenase from the *Clostridium histolyticum* (Sigma; 200 U of collagenase/g of tissue) at 37 °C for 180 min in SCL medium. The digested tissue was filtered through a nylon membrane. Then, cells were pelleted by centrifugation at 600*g* for 15 min. At this stage, supernatant 1 and pellet 1 were subjected separately to further treatment as described (Fig. 1). Supernatant 1 was centrifuged at 20,000*g* for 20 min to sediment all the cell debris, nuclei mitochondria and lysosomes (pellet 2) (Fig. 1). Pellet 2 was discarded while supernatant 2 was centrifuged again at 80,000*g* for 60 min yielding pellet 3 containing MVs (Fig. 1). Cells (pellet 1) were homogenized in 5 ml of sucrose buffer in the presence of protease inhibitor cocktail (Sigma). The homogenate was separated into two parts. The first part was kept for the extraction of membrane homogenates from chondrocytes. The second part was then centrifuged twice at 10,000*g* for 15 min to sediment intact cells, cell debris, nuclei, mitochondria, and lysosomes. To separate the microvillar membranes from the basolateral plasma membranes, the supernatant A (Fig. 1) was supplemented with 12 mM $MgCl_2$, stirred at 4 °C for 20 min to induce basolateral membrane precipitation. It was centrifuged twice at 2500*g* for 10 min to pellet aggregates of basolateral membranes (pellet B) (Fig. 1). Supernatant B was centrifuged at 12,000*g* for 60 min to pellet microvilli (pellet C) (Fig. 1). Protein concentration in these fractions was determined using Bradford protein assay.

2.2. Tissue non-specific alkaline phosphatase activity assay

Tissue non-specific alkaline phosphatase (TNAP) activity was measured using *p*-nitrophenyl phosphate (*p*NPP) as substrate at pH 10.4 and recording the absorbance at 405 nm, using ε *p*NPP = 18.8 mM^{-1} cm^{-1} [16]. Enzyme units are micromole of *p*-nitrophenolate formed per minute per milligram of protein.

2.3. Lipid analyses

Total lipids were extracted from membrane fractions (pellet 3 for MVs, homogenate for membrane homogenates from chondrocytes and pellet C for microvilli) (Fig. 1) according to a modification of the method of Bligh and Dyer [17]. One milliliter of each sample was mixed with 3.75 ml methanol/chloroform (2/1, v/v) in presence of 50 µmol/L of butylhydroxytoluene, vigorously shaken for 1 min, incubated at room temperature for 30 min. Chloroform (1.25 ml) and water (1.25 ml) were subsequently added to the supernatant. The phase separation was performed by centrifugation at 2000*g* for 10 min. The lower organic phase containing the extracted lipids was collected and dried under a stream of N_2. Total lipids were re-dissolved in chloroform and either analyzed by thin

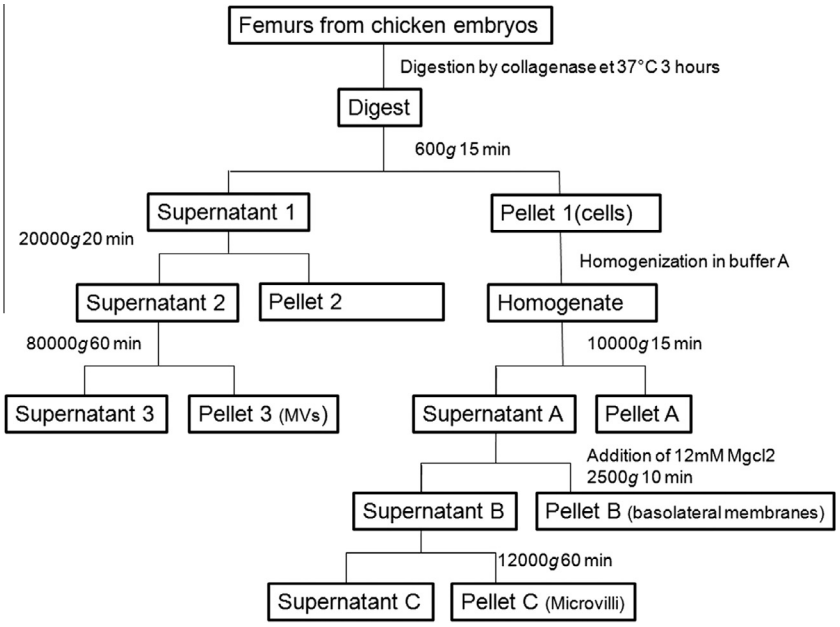

Fig. 1. Schematic overview of the isolation of the matrix vesicles, microvilli and membrane homogenates from chondrocytes.

layer chromatography with the solvent system hexane/diethyl ether/acetic acid (70:30:1, by volume). The silica gel areas corresponding to phospholipids were scraped off and transmethylated for 90 min at 100 °C in 250 µl of toluene methanol (40:60) and 250 µl of BF_3 10% in methanol. The reaction was stopped in ice by adding 1.5 ml of 10% K_2CO_3 (w/v), and FA methyl esters were extracted with 2 ml of isooctane. The derivatives were analyzed by gas chromatography (GC) using an Agilent Technologies chromatograph (model 6890) fitted with a SolGel 1 ms dimethylpolysiloxane capillary column (60 mm by 0.2 mm [inner diameter], 0.25-µm film thickness; SGE Europe, Ltd., France). The oven temperature was set at 80 °C for 1.5 min and increased to 150 °C at 20 °C min^{-1} and then to 250 °C at 2 °C min^{-1}. The temperature was maintained at 250 °C for 10 min before returning to the initial conditions. Helium was used as the carrier gas at 1 ml min^{-1}. The temperatures of the split/split-less injector and the flame ionization detector were set at 230 and 280 °C respectively. The peaks were identified using standard FA methyl esters.

2.4. Statistical analysis

Data are presented as mean ± standard error (SE). For statistical comparison, the *t*-test was employed using SigmaSTAT 3 software. *p*-values under 0.05 were considered to be statistically significant. Three to seven independent measurements (3×40 animals to 7×40 animals) were performed.

3. Results

3.1. Characterization of matrix vesicles and microvilli

Apical microvilli were isolated from chondrocytes according to the method of Jimenez et al. [13] used to obtain microvilli from human placental syncytiotrophoblast. It consisted of the Mg^{2+}-induced precipitation in which non-microvillar membranes were aggregated and could be separated from vesicular microvilli by a low-speed centrifugation (Fig. 1). TNAP is a well known and widely used apical marker of polarized cells [13,14,18]. TNAP activity reached 14.35 ± 0.33 U mg^{-1} and 15.05 ± 0.3 U mg^{-1} for MV and microvillar fractions respectively, whereas the enrichment value of this enzyme was only 2.83 ± 0.44 U mg^{-1} in membrane homogenates from chondrocytes (Fig. 2 A). The distribution of MV proteins closely resembled to that of microvillar proteins but was different from that of membrane homogenates from chondrocyte (Fig. 2B). MV and microvillar protein profiles exhibited similar major bands with apparent molecular weights of: 45, 37 and 31 kDa. Taken together, these findings indicated that the isolated MVs and microvilli were relatively pure as compared with previous findings [8,12,19].

3.2. Fatty acid composition in matrix vesicles, microvilli and membrane homogenates from chondrocytes

FA compositions of MVs and microvilli were similar to each other but were distinctive from those of homogenate membranes. When the relative molar composition (mol in %) was considered, the total saturated and monounsaturated FAs in MVs, microvilli and membrane homogenates from chondrocytes amounted to more than 80% while the total *n*−6 PUFAs were less than 20% , which is consistent with values found in MVs and chondrocytes from tibiotarsal growth cartilage of chick bones [19]. The low level of *n*−6 PUFAs may be related to the involvement of *n*−6 PUFAs in prostaglandin biosynthesis in growth cartilage. The predominant saturated FAs in different fractions were palmitic and stearic acid (16:0, 18:0) (Table 1). Membrane homogenates contained 62% of saturated FAs on average as compared with 48–51% of saturated FAs in MVs and microvilli. The greatest difference in FA content in different fractions was observed in the monounsaturated FAs. The monounsaturated FAs, specifically oleic acid (18:1*n*−9), were higher in the microvilli (18.71%) and in the MVs (27.25%) than of membrane homogenates from chondrocytes (6.48%) (Table 1). We observed that the ratio of *n*−3 to *n*−6 FAs in chondrocyte membranes (0.30) was significantly higher than in matrix (0.06) and microvilli (0.05) membranes. The percentages of 20:4*n*−6 in the MVs (5.61) and in the microvilli (8.08) were significantly higher than that of membrane homogenates (0.12) (Table 1). Since 20:2*n*−6 and 20:3*n*−9. 5,8,11-eicosatrienoic acid (Mead acid, 20:3*n*−9) co eluted [19], we report the combined value. The pooled contents of 20:2*n*−6 and 20:3*n*−9 in MVs (2.011) and in microvilli (1.36) were enriched as compared to that of membrane homogenates (0.74) (Table 1). Membrane homogenates from chondrocytes were enriched in 20:1*n*−9, 18:3*n*−3, 22:5*n*−3 and 22:5*n*−6 as compared to MVs and microvilli.

4. Discussion

It was earlier recognized that FA profiles varied with age of chicks [19,20]. Significant changes of 16:0, 18:0, 18:1*n*−9 and 20:4*n*−6 in chick femurs were observed during aging [20] suggesting that these FAs are involved in bone formation [20]. Our aim was to determine the origin of the enriched FA fractions in MVs and which FAs are essential for the mineralization. We examined and compared FA compositions in MVs and in microvilli extracted from 17-days-old chick embryos, where the FA profile changes are

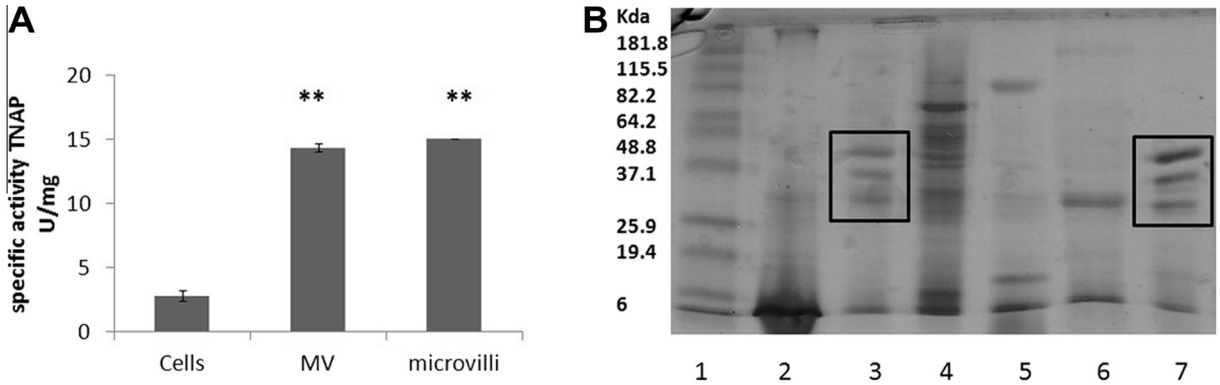

Fig. 2. Characteristics of matrix vesicles, microvilli and membrane homogenates from chondrocytes. (A) Specific TNAP activity in MVs, microvilli and membrane homogenates from chondrocytes. (B) Gel electrophoresis of MVs, microvilli on 10% SDS–PAGE, stained with Coomassie brilliant blue. Lane 1, protein standards; lane 2, 20 µg of supernatant 3; lane 3, 20 µg of MV protein, pellet 3; lane 4, 20 µg of pellet 1 corresponding to membrane homogenates from chondrocytes; lane 5, 20 µg of supernatant C, lane 6; 20 µg of basolateral membrane, and lane 7, 20 µg of Microvilli protein, pellet C.

Table 1
Fatty acid composition of matrix vesicle, microvilli and chondrocytes.

Fatty acid	Matrix vesicle	Microvilli	Cells
14:00	0.47 ± 0.17	0.4 ± 0.2	0.54 ± 0.2
16:00	35.34 ± 1.09	34.44 ± 2.1	42.19 ± 4.09
18:00	13.06 ± 0.56	13.84 ± 0.33	19.06 ± 2.27
22:00	0.225 ± 0.09	0.73 ± 0.38	0.42 ± 0.05
24 00	0.2475 ± 0.14	0.49 ± 0.26	0.44 ± 0.34
16:1*n*−9	2.88 ± 1.1	3.63 ± 1.16	1.43 ± 1.34
16:1*n*−7	2.56 ± 1.19	8.08 ± 3.96	3.65 ± 1.77
18:1*n*−9	27.25 ± 1.15**	18.71 ± 0.59##	6.48 ± 3
18:1*n*−7	0.82 ± 0.69	1.84 ± 1.022	2.14 ± 1.07
18:2*n*−6	7.58 ± 0.75	7.95 ± 0.98	10.67 ± 3.38
18:3*n*−6	0.06 ± 0.02	0.037 ± 0.028	0.07 ± 0.02
18:3*n*−3	0.008 ± 0.005***	0.0078 ± 0.007#	0.08 ± 0.025
20:1*n*−9	0.12 ± 0.052	0.029 ± 0.017#	0.22 ± 0.06
20:2*n*−6 and 20:3*n*−9	2.011 ± 0.32*	1.86 ± 0.15	0.74 ± 0.38
20:3*n*−6	1.87 ± 1.05	0.19 ± 0.11	0.65 ± 0.22
20:4*n*−6	5.61 ± 1.26*	8.08 ± 1.22#	0.12 ± 0.12
20:5*n*−3	0.12 ± 0.06*	0 ± 0#	0.54 ± 0.18
22:4*n*−6	0.51 ± 0.2	0.64 ± 0.36	0.07 ± 0.055
22:5*n*−6	0.26 ± 0.07*	0.093 ± 0.05##	0.58 ± 0.032
22:5*n*−3	0.42 ± 0.21	0.058 ± 0.029	0.97 ± 0.975
22:6*n*−3	0.79 ± 0.37	0.88 ± 0.59	2.25 ± 1.3
Saturated	48 ± 1.29	51 ± 1.45	62.5 ± 6.24
Monounsaturated	33.4 ± 1.6	32.3 ± 3.4	13.9 ± 5.6
PUFA	19 ± 1.87	19.4 ± 2.57	22 ± 1.09
n−3 PUFA	1.07 ± 0.24	0.94 ± 0.56	4.84 ± 0.4
n−6 PUFA	17.6 ± 1.76	18.36 ± 2.24	16.25 ± 0.7
n−3/*n*−6 Ratio	0.06	0.05	0.30

Fatty acid contents were expressed in mol% and the values represent the mean ± SE from three independent determinations (n).
$^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.005$ MV ($n = 7$) vs. chondrocytes ($n = 3$).
$^{\#}p < 0.05$, $^{\#\#}p < 0.01$ microvilli ($n = 3$) vs. chondrocytes ($n = 3$).

expected to be well correlated with bone growth [19,20]. Several enriched FAs in MVs, especially 18:1*n*−9 and 20:4*n*−6 were also found to be enriched in microvilli, in contrast to homogenate membranes where they were less abundant. This suggests that the microvilli incorporated specific FAs for regulating membrane structure, dynamics and permeability, before MVs budding [21]. FA composition in MVs was reminiscent of that of microvilli indicating that the enriched fractions of 18:1*n*−9 and 20:4*n*−6 in microvilli were essential to MVs biogenesis. Oleic acid (18:1*n*−9), represents approximately 20–30% of the total FA content in the MVs and the microvilli. These fractions have higher percentages of 20:4*n*−6, 20:2*n*−6 and 20:3*n*−9 as compared with those in membrane homogenates. This is consistent with higher level of 20:3*n*−9 levels in human fetal cartilage (2.0 ± 0.6% of total FAs) than that in fetal muscle (0.2 ± 0.2%), liver (0.4 ± 0.2%) and spleen (0.1 ± 0.2%) [22,23]. 20:3*n*−9 is endogenously synthesized from oleic acid (18:1*n*−9) [24] which is also enriched in MVs. It was earlier recognized that *n*−6 eicosanoids may regulate chondrocytes differentiation *in vitro* [25]. MVs possess an activated phospholipid metabolism that is regulated independently from that of the cell [26,27]. MV PKC is stimulated by arachidonic acid. In addition, we propose that the enriched fractions of 18:1*n*−9, 20:4*n*−6, 20:2*n*−6 and 20:3*n*−9 accumulated in microvilli are essential precursors for the biogenesis of MVs since they were also found in MVs where they accounted for the most part of FA composition. Due to their relatively high content in MVs and to their selective recruitment within microvilli from where MVs originate 18:1*n*−9, 20:4*n*−6, 20:2*n*−6 and 20:3*n*−9 are essential to maintain and to form MVs in leg bones from chicken embryos.

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