Tumor induced by Moloney sarcoma virus causes periosteal osteogenesis engaging osteopontin, fibronectin, stromelysin-1 and tenascin.

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Excessive bone formation occurring in such conditions as paravertebral ligamentous ossification, hallux osteophytes or some neoplastic tumors, presents a significant problem, both epidemiological and clinical. Since pathogenesis of this disorder is still unclear, we studied its mechanism in experimental model utilizing inducible orthotopic osteogenesis. Periosteal bone apposition stimulated by Moloney sarcoma is characterized by unusually high volume of new bone tissue appearing subperiosteally in the bone adjacent to the tumor. Genes engaged in this growth have not been characterized so far. Here we show the results of mRNA Representation Difference Analysis in Moloney sarcoma, which reveal high expression of four genes coding extracellular matrix proteins: osteopontin, fibronectin, stromelysin-1 and tenascin. These findings suggest that the uncommon dynamics of the Moloney sarcoma –induced osteogenesis depends on high expression of these extracellular matrix proteins.

Key words: orthotopic osteogenesis, stromelysin-1, MMP-3, fibronectin, osteopontin, tenascin, Murine Moloney sarcoma virus, Mu-MSV.

Formation of bone tissue, its turnover and remodeling are controlled by subtle equilibrium of anabolic and catabolic processes. Deficiency in osteogenesis constitutes a major clinical problem in orthopedics, in cases when bone is unable to heal itself, as with segmental bone loss, fracture non-union, failed spinal fusion [1].

Diseases of hyper-active ossification, although having often less remarkable symptoms, have substantial prevalence constituting a significant clinical problem. For example diffuse idiopathic skeletal hyperostosis (DISH) affects over 25% of men and 15% of women ≥50 years of age in North American and European populations [2;3].

Posterior longitudinal ligament ossification (OPLL) and ossification of ligamentum flavum (OFL) (two other types of paravertebral ligamentous ossification[4]) as well as heel spurs and hallux osteophytes [5;6] are widespread examples of orthotopic hyper-ossification. Also, some types of neoplasia display osteogenic properties [7], where persistent inflammation around periosteal membrane leads to formation of these bone malformations.

Bone formation and resorption, whose equilibrium is abolished in these diseases, are dynamic processes with their complex regulation involving cytokines and extracellular matrix proteins (ECM) [8;9]. The mechanisms of bone growth regulation are convoluted and hard to duplicate by experimental models utilizing single gene overexpression [10].

Periosteal osteogenesis can be induced experimentally. Moloney Murine Sarcoma Virus (Mo-MSV) has been shown to transform skeletal muscle into sarcoma. This tumor develops 7–8 days after inoculation and regresses 3 weeks later. Bones adjacent to the tumor increase their width and mass up to 100% at the time of tumor development. Such a massive osteogenic response remains unmatched by other experimental models. Although our first experiments [11–13] were published two decades ago, the nature of this stimulation remains unclear until now. Since Moloney sarcoma is such a powerful trigger for periostal bone formation, we decided to identify genes expressed by this tumor, which could be potentially involved in induction of osteogenesis.

Mo-MSV is a tumorogeneic retrovirus related to Moloney Leukemia Virus (MLV). Its genome contains typical retroviral genes (*pol, gag, env*) and the oncogene *mos* under the control of long terminal repeat (LTR). None of the known Mo-MSV genes have proven osteogenic activity so the effect on bone

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formation is most likely indirect. For the identification of the genes expressed in Moloney sarcoma, we used a molecular approach, where representations of mRNA pools are differentially amplified by PCR. The advantage of this approach is that it discriminates between slightly and heavily increased expressions of mRNA. Only genes highly upregulated are identified by this technique. The following study revealed, that Moloney sarcoma, among other genes, highly expresses mRNA for three potent ECM proteins. Identical tumors in control animals induced hypertrophic periosteal osteogenesis in the bones adjacent to the sarcoma [14].

Materials and Methods

Oligonucleotides. All nucleotides were custom synthesized in the Nucleic Acid Facility at the Thomas Jefferson University, Philadelphia, PA. Sequences of oligonucleotides synthesized were according to Lisitsyn et al. [15]: R-Bgl-24 5'-AGCACTCTCCAGCCTCTCACCGCA-3', R-Bgl-12 5'-GATCTGCGGTGA-3'; J-Bgl-12 5'-GATCTGTTCATG-3', J-Bgl-24 5'-ACCGACGTCGACTATCCATGAACA-3'; N-Bgl-12 5'-GATCTTCCCTGC-3', N-Bgl-24 5'-AGGCAACTGTGCTATCCGAGGGAA-3'

Animals. 4 mice of BALB/c strain were used in the experiment. Mice were propagated in animal facility of the Department of Histology, according to guidelines of Ethical Committee of Medical University of Warsaw. Stock of Mo-MSV was obtained as described [11], aliquoted and stored at -85°C.

Tumor induction. Right thighs of 8 week old mice were injected with 0.1ml of viral stock. 8 days later tumors were detectable at the site of inoculation. At that point 2 mice were sacrificed by cervical dislocation, two others were maintained for the following 20 days and than sacrificed. Mice from day 8 were used to obtain tissue for RNA extraction. Mice from day 28 served as control of the osteogeneic potency of Moloney Sarcoma, which was confirmed by histological analysis of femur adjacent to tumor.

Preparation of cDNA. Tumor tissues form the right shank of the mice inoculated with Mo-MSV 8 days earlier were carefully dissected, weighted, flash frozen on dry ice, finely chopped and lysed in apropriate volume of TRI-reagent (MRC, Inc). Thigh muscle from contralateral (left) legs was dissected and processed in the same way.

RNA was extracted according to manufacturer's protocol. Its concentration and quality were determined by optical density and by electrophoresis on denaturating agarose gel. Polyadenylated RNA was separated on Oligitex beads (Qiagen). This mRNA was used to synthesize doublestranded cDNA (cDNA synthesis kit, Boehringer-Manheim). Efficiency of both steps of the synthesis was monitored by ^{32}P α-dCTP incorporation.

Generation of representations. The detailed protocol of representational difference analysis of cDNA has been published [16]. Briefly: cDNA from transcripts extracted from skeletal muscle was digested with DpnII and ligated (T4 ligase) to adaptors formed by annealed R-Bgl-24 and R-Bgl-12 oligonucleotides. Products of this ligation were further modified in a thermocycler by melting away R-Bgl-12 (3 min, 72°C), filling-in the overhangs with Taq polymerase (5 min, 72°C) and subsequent 20 cycles of amplification (1min 95°C; 3min 72°C). The R-adapters were eliminated by DpnII digestion, purification with phenol/chloroform and ethanol precipitation. The DNA obtained in this way from cDNA isolated from normal tissue formed a driver. The DNA obtained in identical manner from cDNA isolated from sarcoma was later ligated to J-Bgl-12/J-Bgl-24 adapter and formed the tester.

Generation of difference products. Tester and driver were mixed in the ratio 1:50. The mixture was heated to 98°C for 10 minutes in the EEx3 buffer [17] and cooled down to 67°C for 20 hrs to anneal. The hybridized DNA was diluted in TE to a final concentration of 0.1 mg/ml and served as a template for the amplification. In the reaction mix primers and Taq polymerase were omitted. The J-Bgl-12 oligonucleotide was melted away (3min, 72°C), the overhangs were filled with 5U Taq polymerase (5min, 72°C) and J-Bgl-24 primer was added for 10 cycles of amplification (1min, 95°C; 3min, 70°C). The product was purified by phenol/chloroform extraction, ethanol precipitated and digested with Mung Bean Nuclease (New England Biolabs) for 30min at 30°C. After flash heat inactivation (5min, 98°C) and addition of 5U Taq polymerase and J-Bgl-24 primer, the amplification continued for 20 cycles (1min, 95°C; 3min, 70°C). The product of this amplification was phenol/chloroform extracted and isopropanol precipitated giving the first difference product (DP1).

For higher specificity next rounds of hybridization-amplification cycles were performed. The J-Bgl adapters were digested with DpnII, replaced with N-Bgl-12/24 adapters and mixed with the driver at ratio 1:400. Identical procedures to those described above led to isolation of second difference product (DP2). N-adapters were removed from DP2 and replaced with J-adapters. Purified DP2 was mixed and hybridized with the driver at ratio 1:100 000 and the next round of amplification gave the third difference product (DP3).

Analysis of the DP3. The pool of amplified cDNAs in DP3 was DpnII digested and run on TAE agarose gel. EtBr stained bands were cut and DNA was purified with QIAEX II gel extraction kit (Qiagen). DNA purified in this way was cloned into BamHI site of pBluescript KS+. Clones of transformed DH5α cells were grown for mini-preps and double stranded DNA was sequenced with an automated sequenced apparatus (Applied Biosystems). Sequences were analyzed with BLAST program on NCBI database.

Northern Blot. Expression of mRNA coding identified genes was evaluated by hybridization according to general protocol. Briefly, clones representing 3 genes were labeled by nick translation with ^{32}P and used as a probe. RNA from tumor or normal muscle was extracted with TRI-reagent as described above. 10μg were run on denaturing gel (1% Agarose; 1 x MOPS; 0.6 M Formaldehyde) in 1 x MOPS, 0.2M Formaldehyde buffer. Equal loading and the quality of the

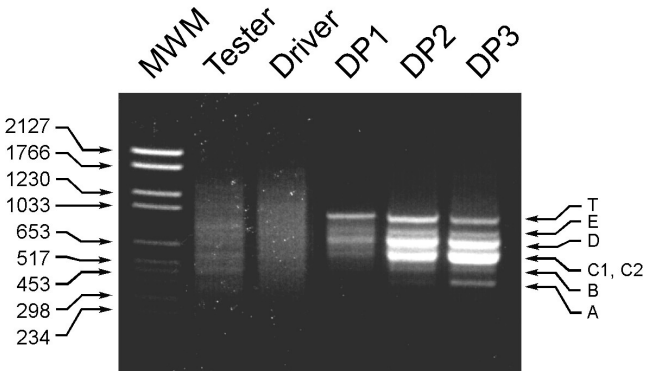

Figure 1. Subtractive hybridization of representations in RDA. Products of consecutive steps of analysis are visualized with EtBr on agarose gel. Tester and Driver were cDNA representations of mRNA expressed, respectively, by Moloney sarcoma or normal skeletal muscle. The first cycle of RDA was subtractive hybridization of tester and driver, followed by PCR which gave the first difference product (DP1). Next rounds of RDA used decreasing ratio of DP1:driver and resulted with products designated DP2 and DP3, respectively.

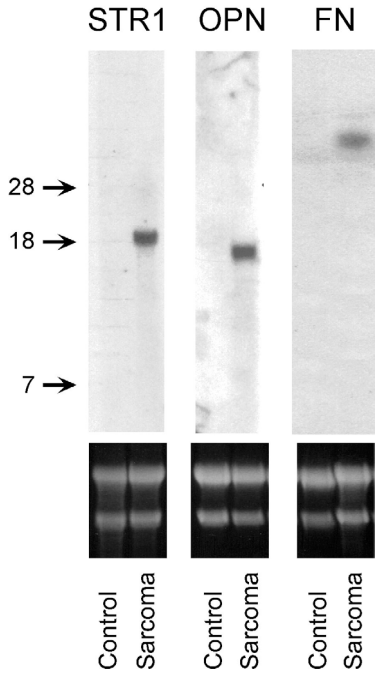

Figure 2. Northern Blot of the RNA isolated from Moloney sarcoma and normal, uninfected skeletal muscle (control). 15 µg/lane of each RNA was run on denaturing gel, then transferred on nylon membrane. ^{32}P-labeled probes for stromelysin-1 (STR1), osteopontin (OPN) and fibronectin (FN) were hybridized, washed and exposed as described. RNA quality and equal loading were verified by EtBr staining.

RNA were verified by EtBr staining of the sample, visualized on the UV-box. RNA was transferred in 10 x SSC by capillary forces onto nylon membrane (Hybond, Amersham). Membranes were washed briefly in 2 x SSC and RNA was 264 nmUV-crosslinked with Stratalinker (Stratagene). Memebranes were prehybridized (5 x SSC; 50 % Formamide; 5 x Denhardt's-solution; 1 % SDS; 100 µg/ml heat-denatured sheared non- homologous DNA (Salmon sperm DNA) and hybridized (5 x SSC; 50 % Formamide; 5 % Dextransulfate; 5 x Denhardt's-solution; 1 % SDS) with the ^{32}P-labeled probes for TN-C, OPN and FN overnight at 42°C. Membranes were washed in decreasing salt concentrations (2-0.1 SSC) and increasing temperature (RT-65°C). Films were exposed with intensifying screens at -80°C.

Results

Representations of cDNA obtained from osteoinductive Moloney sarcoma (tester) and from the control, untransformed tissue (driver) were analyzed for differentially expressed genes. Three rounds of consecutive subtractive hybridizations gave the final product (DP3) highly enriched in representations of genes activated in the sarcoma, and not active in the normal skeletal muscle (Fig.1).

Agarose electrophoresis of DP3 revealed 6 major bands (named: A, B, C, D, E, T) of the size ranging 300–800bp, respectively. Upon closer inspection and more extensive separation on agarose gel, band C turned to be overlaying another band of the similar size (C2). All seven bands were carefully cut from the gel, purified with QIAEX II gel extraction kit and subcloned into BamHI site of pBluescript KS+. 10 clones from each ligation (A through T) were grown for mini-preps, 5 from each group were sequenced. Sequencing results were analyzed with BLASTN software on NCBI web site. The results of the analysis are given in Table 1.

For the verification of the positive clones, RNA isolated from the sarcoma and from the control tissue were hybridized with 3 probes against identified transcripts: OPN, STR-1, FN. All three were strongly upregulated in tumor RNA and absent in the control sample (Fig.2).

To confirm, that the Moloney sarcoma used in the representation difference analysis retained its osteoinductive properties, we analyzed histologically tumors developed in two mice which have been inoculated with Mo-MSV but were not used for RDA. 8µm sections of inoculated shanks stained with H&E show evidence of massive periosteal osteogenesis in the bone adjacent to the tumor (Fig.3).

Discussion

Previous studies have shown that growth factors and structural ECM components can act synergistically to regulate cell growth and differentiation [18]. The analysis of mRNA samples obtained from sarcoma with strong osteoinductive capacity was expected to identify genes which are potentially involved in the process of periosteal bone formation occur-

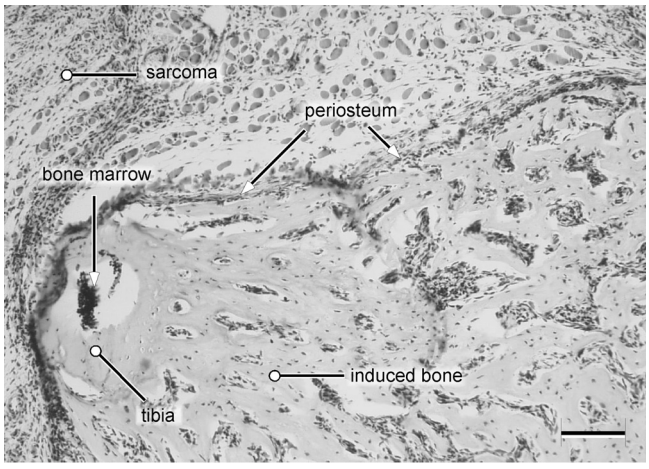

Figure 3. Histological analysis of mouse shank affected by the Moloney sarcoma in the late stage of tumor development. Massive periosteal osteogenesis (induced bone) originating from tibia is the result of tumor-induced stimulation of periosteal membrane of adjacent bone. Moloney sarcoma is in the state of regression. H&E staining, low power. Bar indicates 0.2mm.

ring adjacent to the tumor. Indeed, transcripts of four ECM proteins were found in the Moloney sarcoma: tenascin, osteopontin, stromelysin-1 and fibronectin. All four are known to play a role in bone development, hence are likely to contribute to osteogenesis induced in our model.

Osteopontin (OPN) is a prominent bone matrix protein that is synthesized by osteoblastic cells [19;20], where it facilitates the attachment of osteoclasts to the osteoid (via integrin $\alpha 5\beta 3$ and CD44) and to hydroxyapatite [21]. Elevated expression of OPN is therefore supposed to be certical for bone remodeling. Indeed, OPN is expressed in remodeling areas of the hard callus where it was found in osteocytes, osteoclasts and osteoprogenitor cells [22]. OPN is likely to be a negative feedback regulator for osteoblastic differentiation. Its expression was shown to inhibit pre-osteoblast proliferation rate, caused decreases in expression of osteocalcin and bone sialoproteins and hindered mineral deposition [23]. Also, OPN-/- mice have an attenuated response to inflammation-induced bone and cartilage resorption which further suggests the role of this protein in bone remodeling.

Fibronectin (FN) is an extracellular matrix protein which is critical for bone formation. Moursi and colleagues [24] demonstrated that interactions between cultured fetal calvarial osteoblasts and FN are required for differentiation. Interestingly, FN expression is found only in differentiating osteoblasts [25]. Intact fibronectin is known to prevent apoptosis of proliferating osteoblasts while fibronectin degradation induces their apoptosis [26]. Tumor produced FN forms therefore accommodating environment for osteocytes eventually promoting bone growth. Elevated expression of both: OPN and FN mRNAs were detected in mesenchymal cells derived from skeletal muscles. These cells, under appropriate stimuli, can deposit bone matrix [27].

Our observations of FN mRNA expression are in consent with these reports. It is interesting that FN is expressed simultaneously with the tenascin. As it is discussed below, the mode of TN action is by competition with FN. Therefore we believe that the special distribution of both proteins is tightly regulated in the tumor.

Very strong signal of Stromelysin-1 (STR1) in the northern blot indicates high levels of expression of this protein in the stroma of the Moloney sarcoma. This confirms observations form previous reports, where STR1 or MMP-3 a matrix metalloproteinase was found expressed at high level in the extracellular matrix in fibrous tissue surrounding areas of endochondral ossification in osteophytes, and adjacent to the periosteum of fetal rib bone [28]. Also, osteophytic bone showed varying levels of TIMP-1 – a natural inhibitor of MMPs [29]. Interestingly, in the resorbing bone

Table 1. Identification of DP3 products by BLASTN analysis of sequenced clones. B5 and D5 clones were unidentified due to technical problems.

Ligation	Clone #	Sequence of
	A1	Mo-MSV
	A2	Mo-MSV
A	A3	Mo-MSV
	A4	Mo-MSV
	A5	Mo-MSV
	B1	gp49B
	B2	gp49B
B	B3	gp49B
	B4	Mo-MSV
	B5	–
	C11	Stromelysin-1/ MMP-3
	C12	OPN
C	C13	MDL-1
	C14	Mo-MSV
	C15	Stromelysin-1/ MMP-3
	C21	Mo-MSV
	C22	Mo-MSV
C2	C23	Mo-MSV
	C24	Mo-MSV
	C25	Mo-MSV
	D1	Fibronectin
	D2	Tropomyosin-5
D	D3	Fibronectin
	D4	Fibronectin
	D5	–
	E1	gp49B
	E2	Tenascin
E	E3	Procollagenase
	E4	Mo-MSV
	E5	Mo-MLV
	T1	Mo-MSV
	T2	Mo-MSV
T	T3	Mo-MSV
	T4	Mo-MSV
	T5	Mo-MSV

STR1 expression is not regulated by PTH or Calcitonin [30]. This suggest that expression of this metalloproteinase is not regulated by hormones, hence cannot have a regulatory function on normal bone. The expression of STR1 is in our opinion a manifestation of the particular biology of Moloney saroma.

Tenascins (TN) are upregulated in neoplasia of various origins [31]. In vitro models show, that TN inhibits cell spreading over fibronectin substrate by competing with syndecan-4 for integrin a5b1. This cell-attachment inhibition induces neoplastic cells proliferation [32]. This, subsequently, has also been shown to prevent stress fiber formation in the mechanism involving RhoA inactivation [33]. In glioma cells TN-C up-regulates Wnt signaling and activates MAPK/Erk pathway [34]. Hence, the expression of TN in ECM of Moloney sarcoma may lead to protein kinase activation and, in consequence, to the release of cytokines mediating periosteal bone growth. Although TN might be required for this activation, as it has been localized in developing bone [35], its isolated expression is not sufficient to induce bone formation. Its presence has been found in tumors with no osteoinductive properties [36;37]

Expression of tenascin-C and syndecan-3, a cell surface receptor for growth factors such as fibroblast growth factor 2, have a particular spatial pattern in developing bone. Whereas these two molecules have the same localization and high level of expression in the early stages of osteogenesis in chicken, in the late phase, TN-C expression drops in periosteum [38]. This observation is in agreement with our finding of high TN expression in the samples on the day 8 – at the very early stage of bone induction. Report on high levels of TN-C in metaplastic osteosarcoma [39] seems to support the observation, that this protein is expressed only by highly proliferative bone cells. One of the possible mechanisms of such a high expression of TN in Moloney Sarcoma, could be activation of TGFβ receptor, as it is in the case of osteoblasts. TGFβ regulates TN-C expression by Ets factors [40].

Besides typical ECM proteins, clones of genes related either to the virus itself, or to the leukocytes infiltrating the tumor were identified in our assay. DAP12 is an ITAM-bearing membrane protein that is associated with activating receptors in natural killer cells, granulocytes, macrophages, and monocytes. Myeloid DAP12-associating lectin-1 (MDL-1) is a type II membrane protein that associates with DAP12 [41]. MDL-1 transcript found in the tumor is therefore most likely derived from infiltrating leukocytes. It has been found that MDL-1 and OPN mRNA are both found in dendritic cells [42]. Another gene transcript was identified in our experiment, which is expressed by infiltrating lymphocytes is gp49. It was originally defined as a 49-kDa surface glycoprotein preferentially expressed on mouse interleukin-3-dependent, bone marrow-derived mast cells, which are immature progenitor cells [43]. gp49 is constitutively expressed on cells of the myeloid lineage throughout development, as well as on mature cells. Its expression is induced in vitro on NK cells upon IL-2 stimulation or in vivo by infection with murine CMV [44].

The results presented here give a new perspective for the comprehension of processes which govern bone formation. Our analysis demonstrates that fulminate periosteal bone formation occurs in the presence of osteopontin, fibronectin, stromelysin-1 and tenascin. According to current reports, none of theses proteins alone would stimulate bone formation, but their co-expression as it is found in the stroma of Moloney sarcoma can create the compliant environment for proliferation of osteoblasts. Further studies will tell if co-expression of these proteins is sufficient for stimulation of periosteal osteoblast precursors to the level observed in this model. One can expect that there are additional factors and their spatial and sequential distribution regulating proliferation of bone forming cells.

Availability of parental cells, which differentiate into osteoblasts, could also be one of regulatory aspects of bone formation. To determine if secreted glycoproteins of Moloney sarcoma recruit circulating mesenchymal stem cells, and by those means induce formation of bone, we are currently carrying out experiments in which cell-free bone matrix is implanted in the region of tumor. However, preliminary results obtained so far do not favor this hypothesis (data not shown).

Whether ECM proteins found in our model are critical or only accessory to the chief modulators of bone formation induced by Moloney sarcoma remains to be investigated. Extracellular matrix proteins identified here appear to be engaged in this process either as its necessary components or fine-tuning elements.

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