ACTIVATED N–*RAS* ONCOGENE INHIBITS THE EXPRESSION OF PDGF–BETA RECEPTOR IN MYOBLAST CELLS

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АКТИВИРОВАННЫЙ ОНКОГЕН N–*RAS* ИНГИБИРУЕТ ЭКСПРЕССИЮ РЕЦЕПТОРА PDGF–БЕТА В МЫШЕЧНЫХ КЛЕТКАХ

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By RT-PCR technique mRNA levels of platelet-derived growth factor type-beta receptor (PDGF-beta-R) and MyoD1 in a myoblast cell line carrying human N-ras oncogene have been investigated. After dexamethasone treatment an activation of N-ras and inhibition of the PDGF-beta-R and MyoD1 genes transcription was observed. Those results suggested that cells transformed with the N-ras oncogene fail to respond to PDGF-BB due to the absence of PDGF-beta-R mRNA.
***Key Words*: N-*ras*, MyoD1, platelet-derived growth factor type-beta receptor, myoblast.**

Методом ОТ-ПЦР были исследованы уровни мРНК рецептора PDGF-бета и MyoD1 в линии клеток миобластов, несущих онкоген N-*ras* человека. После обработки клеток дексаметазоном наблюдалась активация транскрипции гена N-*ras* и ингибирование транскрипции генов *PDGF-бета-R* и *MyoD1*. Полученные результаты свидетельствуют о том, что отсутствие ответа к PDGF-BB у клеток, трансформированных онкогеном N-*ras*, связано с угнетением транскрипции гена *PDGF-бета-R*.
***Ключевые слова:* N-*ras*, MyoD1, рецептор фактора роста тромбоцитов, миобласты.**

Ras proteins ($p21^{ras}$), H–, K–, and N–Ras, are members of a family of small G–proteins and are located on the inner side of the plasma membrane. They act as molecular switches through signal network by activation of several cytoplasmic kinases [24]. The guanine binding protein Ras is activated by growth factor receptors and then enter an active GTP–bound state and may function as a growth stimulus [9, 10, 29] or (depending on the cell type) as cell differentiation inducer [4, 5, 11, 37]. Oncogenic mutants of *ras* induce transformation of various cell types causing numerous alterations in processes that affect cellular structure and function [13, 15, 19, 32, 34]. *N–ras* and *H–ras* inhibited myogenic differentiation by supressing the expression of muscle–specific genes such as *mck*, *MyoD* and *Ach* [16, 17, 20].

PDGF–BB is a small protein and binds only to beta–receptor with high affinity [14]. Several investigations were aimed on the elucidation of the influence of $p21^{ras}$ transformation on PDGF–beta–R signaling (reviewed in [26]). EJ–*ras* transformed fibroblasts have diminished PDGF–stimulated PLC activity and also showed 95% reduction in PDGF–stimulated c–*fos* mRNA levels [18]. It was shown [36] that in Kbalb fibroblasts activated *ras* either interacts directly with the PDGF–beta–R to inhibit its kinase activity, or complexes with Syp and/or Grb/2 on the cell membrane and influence another effector which inhibits PDGF–beta–R function. Moreover, transformation of cells by SV40 T/t antigen, v–*src*, v–*abl*, c–Ha–*ras*Val12 or v–*Ki–ras* have been shown to downregulate PDGF–beta–R with significant reduction of corresponding mRNA [22, 26, 28, 30, 35].

Previously we have demonstrated that CO25 myoblast cells carrying mutated human N–*ras* gene failed to respond PDGF–BB stimulation after N–*ras* oncogene activation and are characterized by decreased levels of cytosolic free Ca^{2+}, tyrosine phosphorylation of proteins, and PDGF–beta–R number and protein [33]. In the present work we have investigated the transcription levels of PDGF–beta–R in CO25 cells and have suggested that N–*ras* may affect PDGF–beta–R gene transcription and down–regulate muscle–specific trans–acting transcription factor *MyoD1*.

MATERIALS AND METHODS

***Cell culture*.** The mouse skeletal muscle cell line CO25 carrying the mutated human N–*ras* oncogene [12] was grown in Dulbecco's modified Eagles medium (DMEM) containing 20% of fetal calf serum (FCS), 1% L–glutamine and 100 units/ml penicillin/streptomycin at 37°C in atmosphere of 10% CO_2. To initate differentiation, cultures grown up to 80% confluency were transferred to the same medium containing 10% horse serum (HS) as a fusion promoting medium. The cells were then incubated for five days until the fusion and formation of myotubes were observed. To induce the expression of the N–*ras* oncogene, the cells maintained

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Abbreviation used: Dex – dexamethasone.

in the 10% HS were exposed to 1 μM of dexamethasone (Dex). Phase–contrast observation was carried out using a Nikon TMS inverted microscope fitted with a Nikon F–301 camera.

Murine NIH3T3 fibroblasts were grown in DMEM supplemented with 10% FCS, 1% L–glutamine and 100 units/ml penicillin/streptomycin.

RNA extraction. Total RNA was extracted by acid guanidine–phenol extraction method as described in [7]. The cells were lysed in the denaturing buffer (4 M guanidium thiocyanate, 25 mM sodium citrate, pH 7.0, 0.5% sarcosyl and 0.1 M 2–β–mercaptoethanol); then to the lysate 2M sodium acetate, pH 4.0, water–saturated phenol and chloroform–isoamyl alcohol were added and the mixture was centrifugated at 10000 g at 4 °C. RNA was precipitated with isopropanol, and then resuspended in DEPC treated water.

For RNA extraction, the Swiss albino mouse thigh muscle tissue was minced in ice–cold saline and powdered in liquid nitrogen. After homogenization for 1–2 min, 0.5 g of tissue were lysed in 5 ml of the denaturing buffer and the procedure was performed as described above.

RT–PCR. Prior to the reverse transcription (RT) reaction RNA samples (1 μg) were treated with DNAase. The RT reaction was performed at 42 °C for 60 min using oligo (dT) primers and Moloney murine leukemia virus (M–MuLV) reverse transcriptase (BRL, 200 U/μl). After heat inactivation, specific oligonucleotide primer pairs for PDGF–beta–R, MyoD1 and GAPDH (0.1–1 μM of each primer) were added to the reaction mixture (2mM $MgCl_2$, 1 x PCR buffer and 1.25 U of Taq polymerase, BRL).

The primers were designed according to the mouse PDGF–beta–R, MyoD1 and rat/human GAPDH sequences. The sequences of the primers were as following: PDGF–beta–R: sense – 5'–CCG–GAA–TTC–TCT–TCT–TCA–AGT–CTC–CAA–GTG–C–3' and antisense – 5'–GCC–GTC–GAC–CTG–GCA–GTT–GAG–GTG–GTA–ATC–C–3'; MyoD1: sense – 5'–CCG–GAA–TTC–TCC–CTA–AGC–GAC–ACA–GAA–CAG–G–3' and antisense – 5'–GCC–GTC–GAC–GTG–CTA–TGA–GGA–AAG–GAA–GAG–3'; GAPDH: sense – 5'–CCG–GAA–TTC–AGA–CAG–CCG–CAT–CTT–CTT–(GT)TG–C–3' and antisense – 5'–GCC–GTC–GAC–CTC–CTG–GAA–GAT–GGT–GAT–GG–3'. PCR was performed for 28, 35 or 20 cycles for PDGF–beta–R, MyoD1 and GAPDH, respectively, at 94 ^{0}C for 1 min, 60 ^{0}C for 2 min and 72 ^{0}C for 3 min using a Perkin Elmer Cetus thermocycler.

The amplified PCR products (expected sizes 297 bp, 436 bp and 278 bp for PDGF–beta–R, MyoD1 and GAPDH, respectively) were separated by electrophoresis in 3% agarose gel containing 0.1% ethidium bromide and visualized with the use of CCD camera–based documentation system.

RESULTS

CO25 cells grown as monolayer in the medium with 20% FCS after reaching contact inhibition ceased proliferation and grew in a criss–crossed fashion (Fig. 1, *a*). Cells grown in a medium containing 10% HS with low mitogenic activity displayed significant morphological changes after 4 days of growth, fused and formed long myotubes (Fig. 1, *b*). The addition of 1–2 μM of Dex induced complete morphological transformation within 4–5 days. The cells lost the ability to form myotubes, became fusiformed and grew as groups in a criss–crossed fashion (Fig. 1, *c*).

As an internal control, total RNA was isolated from the cells grown in the fusion promoting medium with or without Dex for 1–4 days, and then subjected to RT–PCR using GAPDH–specific oligonucleotide primers. As an external control, total RNA from mouse tight

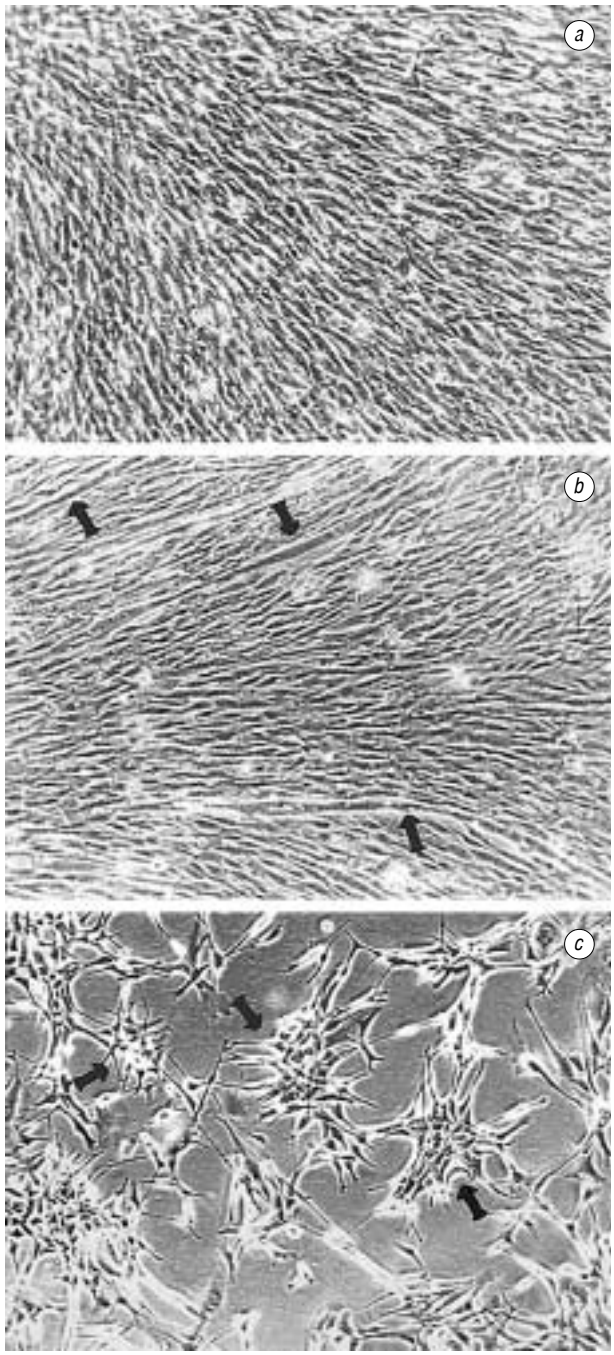

Fig. 1. Morphology of CO25 cells grown in 20% FCS (*a*), 10% HS (b) and 10% HS + Dex (c) for 5 days. Arrows shows the myotubes (*b*) and a clamp of cells (*c*). Phase contrast photographs, magnification 100X

muscle tissue was isolated and subjected to RT–PCR using the same oligonucleotide primers.

As we have shown (Fig. 2, *a*), in CO25 cells grown with 10% HS for 4 and 5 days, PDGF–beta–R mRNA was detected but its level was significantly decreased after Dex–induced activation of N–*ras* oncogene. PDGF–beta–R mRNA expression was also observed in the samples obtained from 3T3 fibroblast cells and mouse muscle tissue used as external controls. The size of RT–PCR product for PDGF–beta–R was close to its expected size (222–350 bp).

MyoD1 mRNA was detected in the cells grown in fusion promoting medium 10% HS for 4 and 5 days, but its level was significantly lower in the cells grown in the presence of Dex for 4 and 5 days (Fig. 2, *b*). MyoD1 mRNA level was found to be very high in mouse muscle tissue, whereas it was undetectable in 3T3 fibroblasts. RT–PCR product for MyoD1 mRNA was close to its expected size (396–460 bp).

RT–PCR products of GAPDH mRNAs were detected in all studied samples and their size was 222–350 bp which is around its expected size 278 bp (Fig. 2, *c*).

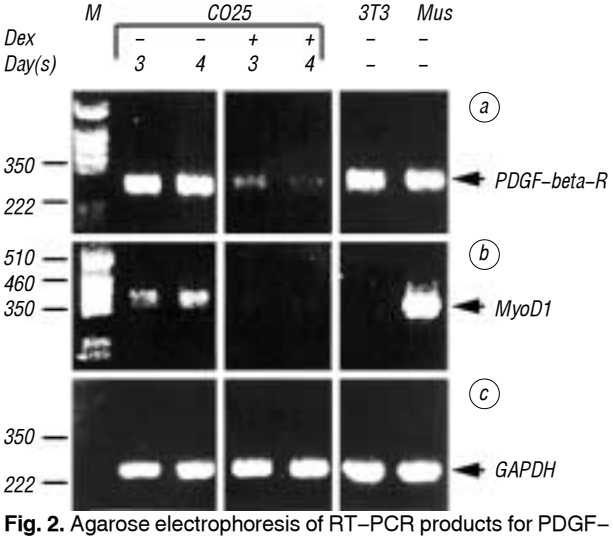

Fig. 2. Agarose electrophoresis of RT–PCR products for PDGF–beta–R (*a*), MyoD1 (*b*) and GAPDH (*c*) mRNAs from CO25 myoblasts grown in the presence (+) or absence (–) of Dex for 4–5 days, NIH3T3 fibroblasts (3T3) and mouse muscle tissue (Mus). Left row show molecular weight markers (b.p.)

DISCUSSION

CO25 cells were obtained by the transfection of C2 mouse myoblast cells with an activated human N–*ras* gene mutated at codon 61 [12]. CO25 cells normally fuse and form multinucleate myotubes after few days of the growth in the medium with low mitogenic activity. The addition of Dex is sufficient to N–*ras* oncogene induction which results in morphological transformation and later — in the changes in *PDGF–beta–R* and *MyoD1* genes expression.

MyoD1 mRNA was detected in the cells grown in fusion promoting medium, but its level was significantly decreased during transformation, indicating the inhibitory effect of N–*ras* activation. Similar data reported in [16, 17] showed that differentiation of *ras*–transfected myoblasts was inhibited by blocking or downregulation of the transcription factors expression.

In CO25 cells after N–*ras* activation the level of PDGF–beta–R mRNA (but not GAPDH mRNA) was significantly decreased. Our results are similar to data of other authors [28, 30, 35] who reported that PDGF–betaR mRNA expression is downregulated in SV40–, v–*src* and v–*abl*–transformed fibroblasts rather than in *ras*–transformed cells. It was shown [8] that SV40–3T3 cells do not produce PDGF in quantities that are sufficient for downregulation of own PDGF–beta–R. c–*myc* protooncogene expression after mitogen or PDGF stimulation caused the suppression of PDGF–beta–R expression on mRNA and protein levels [21]. The similar data were reported in [26] were significant reduction of PDGF–beta–R mRNA levels in v–*Ki–ras* transformed fibroblasts was shown. However, other authors [2, 18, 23] have not found changes in PDGF–beta–R expression levels in $p21^{ras}$–transformed fibroblasts.

Recently the relation of $p21^{ras}$ activation to PDGF signalling have been studied; it was demonstrated that tyrosine phosphorylation of SH2–containing protein–tyrosine–phosphatase Syp (SH–PTP–2) couples Grb2 to PDGFR via its association with Sos1 and Ras, thus providing a mechanism for Ras activation [3]. An indirect association of $p21^{ras}$ with tyrosine kinase–type receptors via some proteins such as Sos, Shc and Grb2 has been reported also [1, 6, 25, 31]. The phosphotyrosine–protein–phosphatase activity in *ras*–transformed NIH 3T3 cells was 2.5 fold higher than in parent cells, indicating that suppression of the PDGFR tyrosine–kinase activity in these cells is mediated via an inhibitory component distinct from the receptor [27]. Howewer, it was reported [36] that the inhibitory activity of Ras is not a phosphatase, because it is not blocked by sodium vanadate.

It is necessary to note, that the downregulation of PDGF–beta–R mRNA was shown in the cells transformed by H–*ras* or K–*ras* genes. The present report describes the inhibition of PDGF–beta–R expression in CO25 myoblast cells after N–*ras* activation. Here, we have demonstrated that in mouse myoblasts the inhibition of PDGF–BB stimulated responses occurs on the level of PDGF–beta–R mRNA expression, suggesting the existence of correlation between the transformed phenotype and reduced expression of PDGF–beta–R.

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