β-catenin signaling and regulation of cyclin D1 promoter in NRK-49F cells transformed by down-regulation of the tumor suppressor lysyl oxidase

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Abstract

Lysyl oxidase is the enzyme that is essential for collagen and elastin cross-linking. Previous investigations showed that lysyl oxidase is down-regulated in many human tumors and ras-transformed cells. Recently, we proved that antisense down-regulation of lysyl oxidase in NRK-49F cells induced phenotypic changes and oncogenic transformation, characterized by p21ras activation and β-catenin/cyclin D1 up-regulation. In the present paper, we examined β-catenin intracellular distribution and its association with E-cadherin. We observed an increased association between E-cadherin and β-catenin in the lysyl-oxidase down-regulated cells during serum starvation. Moreover, we found that β-catenin cytoplasmic and nuclear levels were increased, suggesting a failure of its down-regulation by the APC-GSK-3β system, in particular the GSK-3β phosphorylation of ser-33/37 and thr-41 of β-catenin. Finally, we investigated the mechanisms leading to the observed cyclin D1 up-regulation. We showed that in the antisense lysyl oxidase cells the cyclin D1 promoter was activated through the LEF and the ATF/CRE sites in the proximal promoter. While the promoter activation through LEF is compatible with β-catenin signaling, we investigated the possibility that the CRE-dependent activation might be linked to the down-regulation of lysyl oxidase. In fact, up-regulation of lysyl oxidase in a COS-7 cell model showed a significant diminution of the CREB protein binding to the cyclin D1 promoter, leading to a dramatic inhibition of its activity and a significant down-regulation of cyclin D1 protein level in vivo. Finally, our study describes some major anomalies occurring in lysyl oxidase down-regulated fibroblasts, related to β-catenin signaling and cyclin D1 expression.

Keywords: Lysyl oxidase; Cross-link; Gene promoter regulation; ras; β-catenin; Cyclin D1; TCF; CREB

1. Introduction

Lysyl oxidase (LOX) is the enzyme that catalyzes the oxidative deamination of lysyl residues in collagen and elastin, which allows the formation of cross-links within and between these extracellular molecules [1–4]. This process is critical for their structural and functional features. However, the most intriguing aspect of LOX is its tumor suppressor role. Many investigators have shown that LOX is down-regulated in many oncogene-induced or naturally occurring tumors [5–13]. We showed that microinjection of recombinant mature LOX can block the effects of activated p21ras on Xenopus laevis oocyte maturation, proving some indirect link between LOX and ras pathway [14]. Moreover, we have recently shown that anti-sense down-regulation of LOX in NRK–49F cells induced a tumorigenic phenotype [15]. Indeed, the cells (as-LOX) showed a dramatic phenotype change, increased cell turnover, anchorage-independent growth and tumorigenicity in nude mice. At the molecular level, we detected a significant increase of activated p21ras (GTP-bound form). Moreover, we found an impaired response to PDGF-BB and IGF-1, two important signals regulating cell phenotype, differentiation and proliferation. In our previous paper [16], we described an increase in the levels of β-catenin and cyclin D1 among other features. Both molecules might co-operate in the transformation of...
as-LOX cells. Therefore, we were prompted to investigate more about β-catenin distribution and interaction with its natural partner E-cadherin. At the same time, since β-catenin is directly linked to cyclin D1 expression, we studied some of the molecular pathways responsible for its up-regulation. It is known that β-catenin also exists in a free cytoplasmic pool, which is partially imported into the nuclear compartment and interacts with members of the TCF/LEF-1 transcription factor family, regulating the promoter of several genes [17–23], including cyclin D1. We showed that in as-LOX cell model, the cyclin D1 promoter activity is up-regulated by LEF-1 transcription factor. Moreover, we observed that an equally important role is played by ATF2/CREB factors, which are expressed at higher levels in the nuclear compartment of as-LOX cells. By reproducing a condition opposite to the one occurring in as-LOX cells, that was a COS-7 model transiently over-expressing LOX, we showed that CREB binding is impaired and cyclin D1 expression was down-regulated, suggesting that its regulation might be linked to a LOX-dependent pathway.

2. Materials and methods

2.1. Cell culture

NRK-49F (normal rat kidney fibroblasts), NRK-49F transfected with the empty pcDNA3.1 expression vector (indicated as “vector” or “control”) and as-LOX clones (transfected with pcDNA3.1-antisense LOX) [15] were grown in DMEM/10%FCS, 1% glutamine, 1% non-essential amino acids and antibiotics at 37 °C, 5% CO2 in a humidified incubator. Vector and as-LOX clones were selected with a 400 µg/ml of G418 treatment once a month.

NRK-49F were diluted to 500 µl of total cell lysates, which were cleared for the co-transfected specific renilla luminescence. PSG5-HIS-LOX expression vector has been previously described [14,34]. Luciferase activity was determined by measuring its luminescence in a TD-20/20 luminometer (Turner Designs, Sunnyvale, CA, USA), according to the Dual-Luciferase Assay System (Promega Inc., Madison, WI, USA) directions. The results were normalized for the co-transfected specific renilla luminescence. PSG5-HIS-LOX expression vector has been previously described [14,34].

2.4. Cyclin D1 promoter constructs and luciferase assay

The human cyclin D1 promoter fragments linked to the luciferase reporter gene in the pA3LUC vector were previously described [22,29–31]. We used the full-length −1745, the −1745 TCFm, mutated for TCF/LEF site and the −1745 ATF2m, mutated for CRE/ATF2 site. The TCFm construct carried an AT-CG substitution in position −75 and −74 [22], while in ATF2m the consensus was mutated from 5′-T AAC GTC ACA CGG ACT-3′ to 5′-T cgC GTC cCc CGG gCg-3′ in position −58 [30,32,33]. Luciferase activity was determined by measuring its luminescence in a TD-20/20 luminometer (Turner Designs, Sunnyvale, CA, USA), according to the Dual-Luciferase Assay System (Promega Inc., Madison, WI, USA) directions. The results were normalized for the co-transfected specific renilla luminescence. PSG5-HIS-LOX expression vector has been previously described [14,34].

2.5. Nuclear extracts and cellular fractionation

The cells were collected by scraping with a rubber policeman, after previous wash with PBS. The cells were pelleted at 600 g for 10 min and resuspended in 200 µl/100 mm plate of 20 mM HEPES pH 7.9, 1 mM EDTA,
1 mM dithiotreitol, 0.5 mM PMSF, 1 µg/ml of leupeptin, pepstatin and aprotinin, 1 mM sodium vanadate, 10 mM sodium fluoride and kept at 4 °C for 15 min. The cell suspension was then added with 1/4 vol. of 1% NP40 to obtain a final concentration of 0.2% NP40 and incubated at 4 °C for 15 min. The cell lysate was then centrifuged at 600 g for 15 min. The supernatant (S1) was saved for further processing, while the pellet (P1) mostly containing the unbroken nuclei, was resuspended in 1 vol of BLS (20 mM HEPES pH 7.9, 1.5 mM MgCl2, 0.2 mM EDTA, 0.5 mM dithiotreitol, 0.5 mM PMSF, 80 mM NaCl, 25% glycerol). After extensive mixing, 1 vol of BHS (as in BLS, but containing instead 0.9 M NaCl) was slowly added and incubated at 4 °C for 30 min under moderate shaking. P1 was centrifuged at 16,000 g for 30 min. The supernatant fraction (S2) was collected and used as nuclear extract for gel retardation assay. S1 was then centrifuged at 16,000 g for 30 min, to obtain a partially purified cytosolic (S3) fraction. The quality of the cell fractionation was tested using antibodies against proteins known to be specific of the indicated cell compartments. In details, we detected Na+/K+ ATPase, for the membrane fraction,
GAPDH for the cytoplasmic fraction and Lamin-A for the nuclear compartment. These proteins were also used as housekeeping proteins to normalize the loading in the shown Western blots when necessary. The protein concentration was determined using a Blue Coomassie-based assay [25].

2.6. Electrophoresis mobility shift assay (EMSA)

The assay was performed as previously described [35] using 2–5 μg of nuclear extract proteins in a total volume of 20 μl containing 20 mM Tris–HCl, pH 7.5, 0.1 M NaCl, 0.35 mM dithiotreitol, 0.5 mM EDTA, 0.5 mM PMSF,
Fig. 4. Cyclin D1 promoter activity and effects of inactivating mutations of TCF/LEF and CRE sites in vector and as-LOX cells: luciferase activity induced by −1745 cyclin D1 promoter and −1745 cyclin D1 promoter carrying a mutation for TCF and ATF2 site transfected in vector and as-LOX cells. The experiment represents the average±S.D. of at least 3 experiments performed in triplicate. The promoter activity was expressed as luciferase/renilla ratio. The significance of the difference between the activities of the different constructs in vector and as-LOX cells was evaluated by t-test analysis. The transfection and luciferase assay conditions are described in Materials and methods.

Fig. 5. EMSA for cyclin D1 promoter TCF site using vector and as-LOX cell nuclear extracts A, EMSA using vector and as-LOX cell nuclear extracts incubated with the γ[32P] labeled double-strand cycD1-TCF oligonucleotide. The competition of the DNA binding was obtained using a 100-fold excess of unlabeled double-stranded oligonucleotide competitor to the incubation mixture. Where indicated, the nuclear extracts were pre-incubated with the specified antibody for 30 min at 4 °C and then used for the gel retardation assay. The antibodies were the same used in Western blot analysis and are indicated in the relative figure legends.

3. Results and discussion

Previously, we showed that NRK-49F cells undergo transformation upon stable transfection with an anti-sense LOX expression vector (as-LOX cells) [15]. Among the features displayed by the as-LOX cells, we noticed a looser attachment to the substrate, compatible with their ability to grow in soft-agar, and a higher tendency to grow in tight small groups [15]. Moreover, the as-LOX cells were able to survive and proliferate at a normal rate in the absence of serum for at least 48–72 h, suggesting that some cell cycle-regulatory mechanisms may be constitutively altered. While the increased level of active p21^ras may explain the oncogenicity of these cells [36–39], the possibility exists that some other mechanisms may be involved in the induction of the proliferation in synergy with ras. The increased cell–cell adhesion exhibited by the as-LOX cells suggested the possibility of an abnormality in E-cadherin/β-catenin signaling. We have previously demonstrated by immunofluorescence an increase of β-catenin in as-LOX cells [16]. In Fig. 1, we confirmed those results by Western blot on a total cell lysate (Fig. 1A) and a cell fractionation analysis (Fig. 1B). In Fig. 1A, we showed an overall increase of the total β-catenin in the as-LOX cells. Fig. 1B shows the levels of β-catenin in the membrane, cytosol and nuclear fractions. It is evident that most of the increase in β-catenin levels occurred in the cytosolic and nuclear compartments. Like the following experiments, this shows a typical result obtained with a specific clone of as-LOX cells, but similar results were confirmed with other as-LOX clones [15]. A variety of components of the APC/Axin/GSK-3β/Akt signal transduction pathway may be altered to cause the observed β-catenin deregulation. One possible
candidate is GSK-3β, which is known to target β-catenin to the ubiquitination pathway by specific serine/threonine phosphorylations. One possibility was that in as-LOX cells, GSK was inactivated by a specific serine phosphorylation, probably AKT-dependent, but this was not the case (data not shown). Nevertheless, the GSK-dependent phosphorylation of β-catenin at serine-33/37 and threonine-41, which is responsible for its down-regulation, was virtually absent in
as-LOX clones, except for a transient induction after 24 h of exposure to 10% FCS (Fig. 1D). Considering the peculiar growth characteristics of as-LOX cells [15,16] and the abnormal β-catenin distribution, we decided to analyze the pattern of interaction between β-catenin and E-cadherin, since they are among the main regulators of cell–cell interaction. Interestingly, we found that their interaction seemed to be influenced by the growth conditions. Fig. 2 shows that during the starvation the co-immunoprecipitation of E-cadherin using an anti-β-catenin antibody is much higher in as-LOX cells than in the control ones, while the pattern is inverted after 48 h of exposure to 10% of calf serum. This is mostly due to a dramatic serum-induced increase of the binding between the two molecules in the control cells. On the other hand, as-LOX cells appeared almost unaffected by the presence of serum. Similar results were obtained by immunoprecipitating E-cadherin and detecting β-catenin (data not shown). The most obvious explanation would be a different degree of phosphorylation of these molecules during the serum-induced growth, which would influence their interaction. Many reports have shown that E-cadherin and β-catenin interaction is regulated by multiple serine/threonine and tyrosine phosphorylations from several growth factor or oncogenes [40–43]. Therefore, we tested the phosphorylation status of β-catenin and E-cadherin. While we could not detect any difference in tyrosine phosphorylation (data not shown), the immunoprecipitation of β-catenin brought down a 115-kDa serine phosphorylated protein that was in higher concentrations in as-LOX cells when starved and in the control cells when grown in presence of serum (Fig. 3A top). Based on its molecular weight, we concluded that the phosphorylated band was not β-catenin, however, there was a possibility that it could have been E-cadherin. In fact, a Western blot of the same filter with anti-E-cadherin antibody showed that the E-cadherin band overlaid the phosphorylated band (Fig. 3A lower). Interestingly, the highest phosphorylated status of E-cadherin corresponded to an increased co-immunoprecipitation with β-catenin (see Fig. 2). The ratio between total and phosphorylated E-cadherin was between 2 and 3 when the amount of co-immunoprecipitated E-cadherin was the highest (starved as-LOX cells and FCS control cells). Conversely, the ratio was above 7 where the phosphorylation is barely detectable (Fig. 3B). In the starved as-LOX cell immunoprecipitation, we also detected a phosphorylated band above 120 kDa that could not be identified, but we ruled out p130cas by specific antibody detection (data not shown). Whether E-cadherin serine phosphorylation plays a role in the interaction with β-catenin is difficult to say, but again it does not affect as-LOX cells, just like many other physiological stimuli (see PDGF, FGF in [44]).

An increased nuclear signaling of β-catenin has been reported to up-regulate cyclin D1 among other genes [22,23,45,46] by activating the TCF/LEF transcription factors. We showed a 2.5-fold increase of cyclin D1 in as-LOX cells as compared to the vector clones [16], which prompted us to study the regulation of its promoter in this cell model. We found that the activity of the full-length human cyclin D1 promoter linked to the luciferase reporter gene was induced from 6- to 8-fold in the as-LOX cells, as compared to the control vector cells (Fig. 4, −1745), which is also consistent with the endogenous cyclin D1 levels [16]. Since CREB has also been previously indicated as an important regulator of cyclin
D1 promoter [33] and is often increased in cells transformed by ras oncogenes, we decided to assess the individual roles of these two transcription factor families in the up-regulation of the cyclin D1 in as-LOX cells. Thus, we performed experiments in which cyclin D1 promoter/luciferase constructs containing inactivating mutations in the TCF/LEF site at position −81 [22] and in the CRE sequence located at −58 [30,32,33] were transfected into the vector and as-LOX cells. As seen in Fig. 4, mutations of TCF/LEF (−1745 TCFm) or ATF2/CRE (−1745 ATF2m) sites reduced the activity of the cyclin D1 promoter, by 30 and 70%, respectively, in the vector cells and by 95 and 99% in the as-LOX cells. Although both transcription factors seem to affect cyclin D1 promoter activity, our results suggested a more important role for CRE/ATF2 elements in both cell lines, and in as-LOX cells both seem to regulate the promoter activity in a stronger way, considering the much higher basal activity. An Electrophoresis Mobility Shift Assay (EMSA) was next used to investigate whether the gene reporter results were backed-up by a consistent variation in the binding of these two transcription factor families to the cyclin D1 promoter. For this purpose, we used oligonucleotides carrying either TCF/LEF or CRE/ATF2 consensus sequences from cyclin D1 promoter region. When TCF/LEF probe was tested with the as-LOX nuclear extracts, the specific DNA–protein complex was increased at least 3 times as compared to the vector cells, while a probe containing the same mutation as in the cyclin D1 luciferase constructs abolished the formation of the complex (Fig. 5A). Moreover, the complex was partially abolished by anti-LEF-1, which also produced a super-shift of the complex, but was not affected by anti-TCF-4, antibodies (Fig. 5B). A Western blot of vector and as-LOX nuclear cell extracts revealed an increase of LEF-1 nuclear level in as-LOX cells, while TCF-4 was actually down-regulated (Fig. 5C–E). Similar results were obtained in EMSA for the binding to the CRE element, where the
formation of the complex was increased from 3 to 4 times in the as-LOX cells, and was specifically displaced by an excess of cold probe, but not by an unrelated probe (Fig. 6A). Specific antibodies against CREB and ATF2 induced a clear super-shift of the band, indicating that both factors were present in the detected DNA–protein complex (Fig. 6B). The Western blot for CREB and ATF2 also confirmed a 4- to 5-fold increase in the nucleus of as-LOX cells (Fig. 6C–E) for both transcription factors.

Since CREB regulation is not described as a consequence of β-catenin signaling, we tested the possibility that it could be an effect linked to LOX down-regulation. Therefore, COS-7 cells transfected with recombinant mature human LOX, which we have previously shown is also expressed in the nuclear compartment [34]. EMSA experiments were performed with CRE probe using the nuclear extracts from COS-7 cells transfected with the empty vector (pSG5) or the vector carrying LOX mature protein CDS (pSG5-His-LOX). As shown in Fig. 7A right panel, in the cells over-expressing LOX, there was a significant decrease in the CREB/ATF2 complex as compared to the vector-transfected cells. These data are the opposite of the ones obtained in as-LOX cells, where LOX was down-regulated (see same figure, left panel) and, therefore, consistent with a role of LOX in the regulation of CREB signal transduction. Fig. 7C confirms the different levels of LOX in NRK-49F, as-LOX cells and in COS-7 cells un-transfected or transfected with recombinant mature His-LOX. Moreover, as shown in Fig. 7B, the level of luciferase driven by the cyclin D1 promoter was inhibited by approximately 80% in COS-7 cells transfected with LOX, as compared to the cells transfected with the empty vector. Surprisingly, the activity of the −1745 ATF2/CREmut was induced 2-fold by LOX over-expression. A possible explanation for the latter effect is that LOX, in some way, impaired the binding of ATF2/CRE factors, which accounts for the observed inhibition, but at the same time mediated an activation through different sites, which becomes dominant in the absence of CRE consensus sequence. In our previous work, we showed also that LOX increased COL3A1 promoter activity probably by increasing Ku proteins binding to a site highly homologous to NRE1 [47,48] (the increased Ku binding in COS-7 expressing LOX is indicated also in Fig. 7). Analyzing the cyclin D1 promoter, we actually found several regions very similar to the COL3A1 promoter sequences regulated by LOX and they showed also some homology with NRE1 (Fig. 7C). To confirm that this LOX-dependent regulation also occurs in vivo, we analyzed the cyclin D1 protein level in the same COS-7 cells expressing or not the recombinant mature LOX. The results shown in Fig. 8A (upper panel) indicated that COS-7 transiently transfected with LOX expressed lower levels of cyclin D1 (about 55% of the control, as calculated by densitometry, Fig. 8B). Although the inhibition is less than what we obtained in the gene reporter experiments, it is significant, considering that the percent of transfection efficiency usually does not achieve more than 10–20%. Interestingly, we also observed (Fig. 8A, lower panel) that LOX over-expression did not affect the overall expression of β-catenin (which was used in the experiment also as internal control for the protein load). Although our data are not conclusive, they suggest some specific LOX-dependent regulation of the cyclin D1 promoter through alterations of ATF2/CREB signaling.

In summary, our study demonstrated that the loss of LOX function in normal kidney fibroblasts resulted in important changes in critical cell adhesion mechanisms. Although the literature shows that LOX tumor suppressor role is present in both epithelial and fibroblasts cells [8–10,49–51], it should be kept in mind that our results refer to a fibroblast cell model, where the adhesion mechanism

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**Fig. 8. Effects of recombinant mature LOX over-expression on cyclin D1 expression in COS-7 cells.** A, Western blot analysis of whole cell lysates from COS-7 mock transfected with empty pSG5 vector (control) or with pSG5-His-LOX vector (LOX) using the anti-cyclin D1 polyclonal antibody (06-137; Upstate Biotechnology). In the lower panel, it is shown, as internal control, the expression of β-catenin (same antibody as in Fig. 1), which is not modified. B, plot indicating the optical densities of the western blot bands as described in Fig. 1. Western blot and transfection conditions are indicated in Materials and methods.
might work differently than an epithelial cell. We described an increase of the β-catenin-free-pool. Surprisingly, the β-catenin deregulation did not affect its binding to E-cadherin, which was even higher in critical growth conditions as during prolonged starvation. A specific serum-dependent serine phosphorylation on E-cadherin might be physiologically responsible for this regulation, but it did not affect as-LOX cells. We showed that the activation of β-catenin signaling through LEF could be in part responsible for the increased levels of cyclin D1 and cyclin D1 promoter activity. However, we also found an activated CREB/ATF2 pathway that in large part accounted for cyclin D1 up-regulation. We cannot rule out that this might be mediated by the constitutively activated p21ras present in the LOX-down-regulated cells, as elsewhere described [23]. Nevertheless, the ability of LOX over-expression to repress the pattern of CREB/ATF2 binding to the cyclin D1 promoter, together with cyclin D1 down-regulation at the protein level, also suggested an indirect involvement of LOX in cyclin D1 promoter regulation. Studies are in progress to define the mechanisms by which LOX exerts its effects on this and other promoters [34].

Besides our observation of LOX effects on COL3A1 promoter probably through the Ku factor [34], a recent report described a LOX interference on the transcription factor NF-κB pathway [52]. Unfortunately, like in our case, the authors could not explain the mechanisms underlying those effects. We believe that LOX exerts its effects through some unknown intracellular target/partner. Previously, we have pointed out the possibility that LOX might target histone H1 [53]. The important position of H1 in the regulation of gene transcription [54–57] could explain many of LOX effects on gene promoters and transcription factors. We cannot be sure yet that histone H1 is the link between LOX and cyclin D1 promoter regulation, but efforts are being made to test this hypothesis in vitro and in vivo. Taken together, our data show that NRK cells transformed by LOX down-regulation presented an activation of β-catenin signaling leading to up-regulation of cyclin D1. This phenomenon could be an associated cause of the cell transformation, which is usually never achieved by ras oncogene alone. While this is certainly the results of complex signaling derived from the absence of a tumor suppressor (previously, we showed that all the clones down-regulated for LOX showed the same features, although to different extent [15,16]), we showed that indirectly the levels of LOX can affect the promoter activity and protein level of cyclin D1 through CREB. An earlier report suggests that cAMP can regulate LOX expression [58], therefore LOX effects on CREB binding and expression level might be part of a more complex cross-talk between the two pathways. Hopefully, further studies for the identification of new intracellular LOX targets, and the investigation of the signal pathways affected by LOX will help understanding its role in the oncogenic transformation process.

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