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Inhibition of Collagen Fibrillogenesis by Cells Expressing Soluble Extracellular Domains of DDR1 and DDR2

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Collagen fiber assembly affects many physiological processes and is tightly controlled by collagen-binding proteins. However, to what extent membrane-bound *versus* cell-secreted collagen-binding proteins affect collagen fibrillogenesis is not well understood. In our previous studies, we had demonstrated that the membrane-anchored extracellular domain (ECD) of the collagen receptor discoidin domain receptor 2 (DDR2) inhibits fibrillogenesis of collagen endogenously secreted by the cells. These results led to a novel functional role of the DDR2 ECD. However, since soluble forms of DDR1 and DDR2 containing its ECD are known to naturally exist in the extracellular matrix, in this work we investigated if these soluble DDR ECDs may have a functional role in modulating collagen fibrillogenesis. For this purpose, we created mouse osteoblast cell lines stably secreting DDR1 or DDR2 ECD as soluble proteins. Transmission electron microscopy, fluorescence microscopy, and hydroxyproline assays were used to demonstrate that DDR ECD expression reduced the rate and quantity of collagen deposition and induced significant changes in fiber morphology and matrix mineralization. Collectively, our studies advance our understanding of DDR receptors as powerful regulators of collagen deposition in the ECM and elucidate their multifaceted role in ECM remodeling.

Keywords: DDR1; DDR2; collagen fibrillogenesis; TEM; ECD

Introduction

Collagen fibrillogenesis, the assembly of collagen fibers, is a critical process in the development, maturation, and repair of mammalian tissue. Alterations in the structure and amount of deposited collagen fibers can greatly alter the integrity of the whole tissue. Even a single point mutation in collagen type I can severely compromise the strength of cortical bone tissue leading to osteogenesis imperfecta.[1] Further, the interaction between collagen-binding proteins and collagen molecules during fibrillogenesis can promote significant alterations in the resulting collagen fiber structure and subsequent extracellular matrix (ECM) remodeling.[2,3] For example, soluble collagen-binding proteins such as decorin, biglycan, fibronectin, and vitronectin are thought to play a significant role in the process of collagen fibrillogenesis and bone mineralization due to their interaction with collagen molecules.[4]

The collagen-binding membrane proteins discoidin domain receptors (DDR1 and DDR2) are transmembrane receptors belonging to the family of receptor tyrosine kinases and have been studied for ECM remodeling in atherosclerosis,[5–7] osteoarthritis,[8–10] and several malignancies.[7] It is

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Abbreviations used: DDR, discoidin domain receptor; ECD, extracellular domain; ECM, extracellular matrix; KD, kinase deficient; TEM, transmission electron microscopy; HP, hydroxyproline; FITC, fluorescein isothiocyanate; SMC, smooth muscle cell.

well established that activation of the DDR1 and DDR2 kinase domain up-regulates the expression of various matrix metalloproteinases[5,11] and alters the biosynthesis of collagen.[5] The extracellular domain (ECD) of DDRs is known to be necessary and sufficient for its interaction with collagen.[11] Besides the full-length receptor, the DDR1 ECD is also found in five distinct isoforms[12] and as a shed protein in the ECM.[13,14] Several protein[5] and mRNA[15–17] species consisting of the DDR2 ECD have also been observed *in vivo*. However, the functional roles of these ECDs of DDRs lacking their kinase domain are not well understood. We had previously elucidated that DDR1 ECD[18] and DDR2 ECD[19] inhibit collagen fibrillogenesis *in vitro* when used as purified proteins. Further, we have recently demonstrated that the DDR2 ECD when anchored on the cell surface preserves the capacity to inhibit collagen fibrillogenesis independent of its kinase activity.[2] It is therefore likely that the expression of soluble ECD of DDRs by cells such as those found in the shedding of DDR1 ECD[12] may play an important role in matrix remodeling.

The fibrillogenesis process of collagen is understood to initiate in the extracellular space near the plasma membrane where secretory vesicles form regions of deep invagination.[20] However, it is not clear how and when collagen-binding proteins interact with collagen molecules during fibrillogenesis or to what extent membrane-bound *versus* soluble collagen-binding proteins affect the collagen fibrillogenesis process by cells. In this study, we seek to elucidate the alterations in collagen fibrillogenesis arising due to soluble DDR1 and DDR2 ECDs secreted by the cells and compare the results with our previous findings utilizing the kinase-deficient, membrane-bound DDR2 ECD (DDR2/-KD). Similar to our previous study, we created stably transfected mouse osteoblast cell lines to express DDR1/ECD or DDR2/ECD as a soluble protein. We utilized a number of ultrastructural and biochemical analyses to elucidate how alterations in the collagen matrix, due to DDR ECDs, affects collagen fibrillogenesis and matrix mineralization.

Results

Characterization of stable cell lines

We used mouse pre-osteoblast cell line MC3T3-E1 to ascertain the effects of cell secretion of DDR1/ECD and DDR2/ECD on collagen fibrillogenesis by the cells. Based on previous studies by us[2] and others[21] these cells secrete and form well-defined collagen fibers in the ECM over a period of one to several weeks. These cells were stably

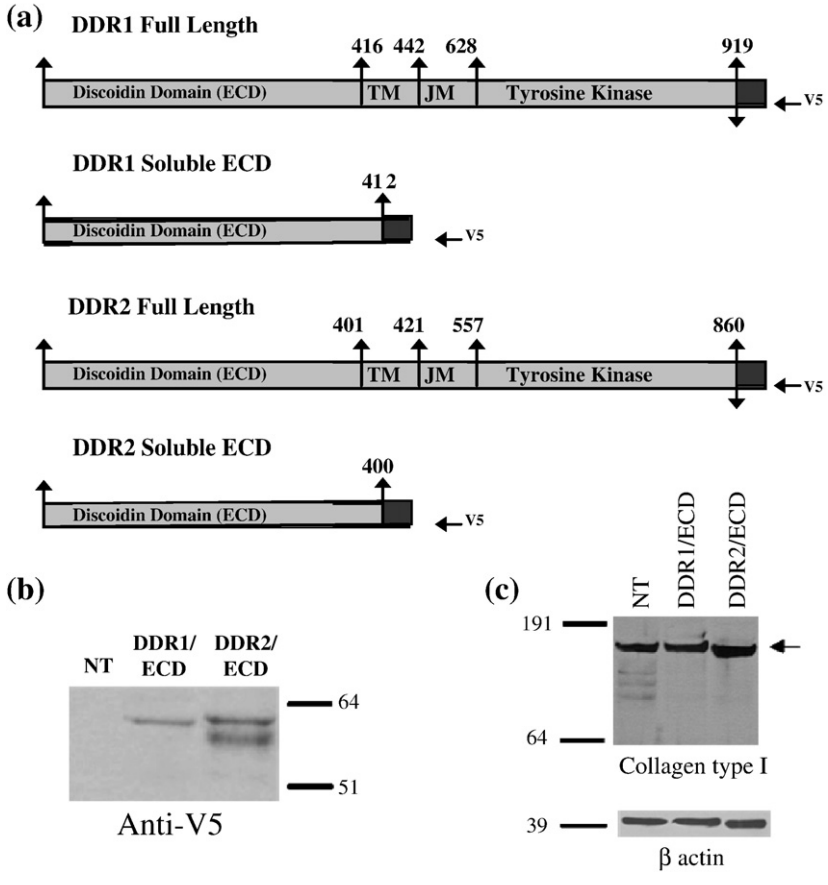

Fig. 1. Creation of stable cell lines expressing the recombinant protein DDR1/ECD and DDR2/ECD. (a) A schematic representation of the V5 His-tagged full-length mouse DDR1 and DDR2 and the V5 His-tagged soluble ECD proteins, DDR1/ECD and DDR2/ECD. (b) Verification of DDR1/ECD and DDR2/ECD in conditioned media of stable cell lines. Western blot analysis using anti-V5 antibodies was performed on conditioned media from each cell line as indicated. Presence of DDR1/ECD and DDR2/ECD was observed at their expected molecular masses, slightly under 64 kDa as a single band (for DDR1/ECD) and as a doublet for DDR2/ECD. (c) Western blotting of whole-cell lysates from nontransfected or stably transfected cells (as indicated) shows no significant difference(s) in collagen expression in the various samples.

transfected with V5-epitope-tagged DDR1/ECD and DDR2/ECD expression constructs (Fig. 1a) to ensure long-term secretion of these proteins in the ECM. The expression of DDR1/ECD and DDR2/ECD in our transiently transfected (data not shown) and stable cell lines was confirmed using Western blotting (Fig. 1b) of the conditioned media obtained from these samples. DDR2/ECD was expressed as a doublet in Western blots, consistent with it being a glycosylated protein as observed by other investigators.[5,22–24] Cells stably transfected with DDR1/ECD or DDR2/ECD did not show alterations in collagen protein expression as shown by Western blotting of whole-cell lysates of these samples (Fig. 1c).

DDR ECDs disrupt the structure of collagen fibers

To determine if secretion of DDR1/ECD and DDR2/ECD by the cells affects the structure of collagen fibers assembled by the cells in the ECM, we employed ultrastructural morphological analysis using transmission electron microscopy (TEM) of cell cultures. Figure 2 shows the collagen fiber morphology of nontransfected DDR1/ECD and DDR2/ECD stable cell lines at 3 weeks of culture (similar fiber morphology was observed for all time points of culture duration). Collagen fibers formed in the ECM of nontransfected cells have well-defined banded structures exhibiting D-periodicity. Nontransfected samples exhibited a D-periodicity average of 61 ± 5 nm ($n = 100$) across weeks 1 through 3. In contrast, the collagen fibers in the ECM of both DDR1/ECD and DDR2 ECD stable cell lines exhibited poorly formed fiber morphology lacking the characteristic D-periodicity at all time points.

DDR ECDs affect collagen fiber diameter

Fiber-diameter analysis was conducted on the collagen fibers present in the ECM of nontransfected, DDR1/ECD, and DDR2/ECD cell lines (Fig. 3). The TEM micrographs clearly show larger fiber diameters for the native cells as compared to cell lines overexpressing DDR1/ECD or DDR2/ECD. While nontransfected cells exhibited an average fiber diameter of 55 nm, the average fiber diameters for DDR1/ECD and DDR2/ECD samples were 23 and 26 nm, respectively. This disparity in collagen fiber diameter between native cells and DDR1/ECD and DDR2/ECD cell lines was consistent with other time intervals as well (Fig. 4). DDR1/ECD samples had an average fiber diameter in the range of 23.1 to 27.6 nm across the 4-week time intervals, while DDR2/ECD samples exhibited fiber diameters ranging from 23.8 to 28.4 nm. The nontransfected cell cultures had consistently larger fiber diameters, producing average fiber diameters of 45 to 55 nm across the 4-week time intervals. These observations confirm that DDR1/ECD and DDR2/ECD expression in the ECM reduces collagen fiber diameter. Standard deviation of the average fiber diameter was observed to be slightly higher in the nontransfected cell samples (9–10 nm) as compared to those of DDR1/ECD or DDR2/ECD samples (5–7 nm). Further, the cross section of collagen fibers present in DDR1/ECD and DDR2/ECD samples often show fused fibers with noncircular cross sections, while the nontransfected samples exhibited well-separated fibers with distinct circular cross sections.

DDR ECDs inhibit the kinetics of collagen fibrillogenesis

To ensure that the inhibition of collagen fibrillogenesis observed in the DDR ECD stable cell lines was due to the expression of DDR1/ECD or DDR2/ECD and not due to differences in the amount of collagen being secreted by these cells, fluorescent microscopy was used to observe how DDR/ECD altered fibrillogenesis of exogenously added collagen (Fig. 5). Nontransfected cells and cells transiently or stably transfected with DDR1/ECD and DDR2/ECD were cultured in the

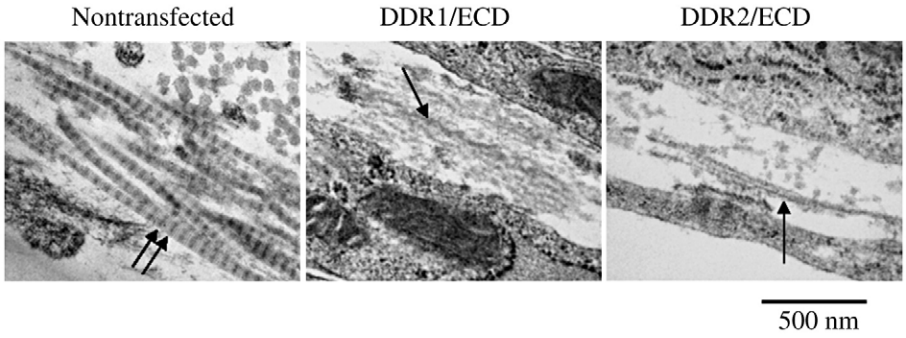

Fig. 2. TEM micrographs showing ultrastructure of collagen fibers assembled in the ECM of 3T3 cells after 3 weeks of culture. Nontransfected samples show well-formed collagen fibers with defined D-periodic structure (double arrow). This banded structure of collagen fibers is hindered in DDR1/ECD and DDR2/ECD stable cell lines (indicated by single arrows) for all time points (week 3 is shown). Magnification of micrographs is 50,000×.

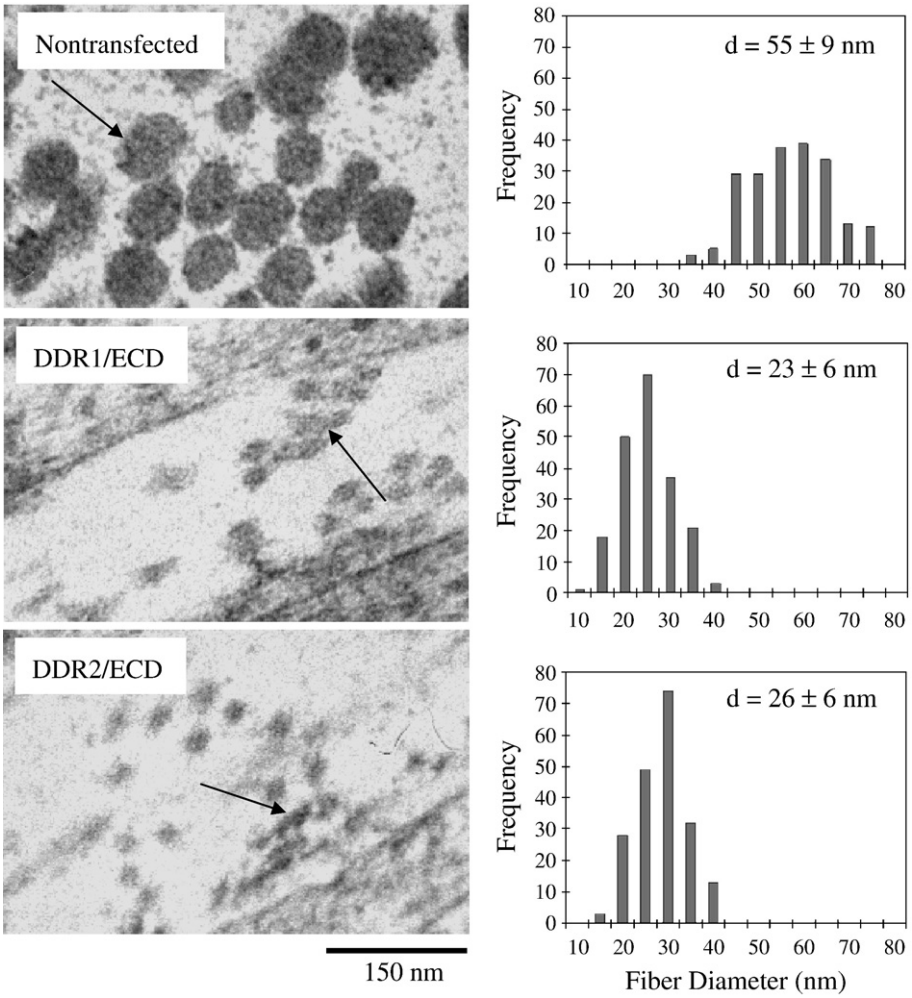

Fig. 3. Collagen fiber diameter is affected by overexpression of soluble DDR1/ECD and DDR2/ECD in the ECM of cultured cell lines. The average fiber diameters for both DDR1/ECD and DDR2/ECD samples are notably smaller than those of the nontransfected samples (NT). *d* is the average diameter (nm) obtained by measuring 200 fibers from each sample type after 2 weeks of culture. The fiber cross sections of DDR1/ECD and DDR2/ECD display fused fibers (arrow), while the nontransfected samples show characteristic circular cross sections (arrow). Magnification of micrographs is 50,000×.

absence of ascorbic acid, and monomeric collagen I labeled with fluorescein isothiocyanate (FITC) was exogenously added for various time intervals. The nontransfected cells show evidence of fiber formation as early as 1 h after addition of collagen, with continued growth in collagen fibrillogenesis throughout the 12-h period. During this same period, there was negligible fiber formation in the cell samples stably or transiently transfected with DDR/ECDs.

DDR ECDs decrease collagen deposition in ECM

Our TEM analysis (Figs. 2 and 3) and fluorescence microscopy data (Fig. 5) indicate suppression of fibrillar collagen content in cells overexpressing DDR1/ECD and DDR2/ECD. To ascertain and quantify the total collagen content in the adherent ECM, we carried out the hydroxyproline (HP) assay, a biochemical technique used for routine measurement of collagen content in tissue specimens.[2,25] Nontransfected cells and DDR1/ECD and DDR2/ECD stable cell lines were cultured in the presence and absence of ascorbic acid for 1 and 2 weeks and the adhered cell layer was subjected to HP analysis (Fig. 6). Little difference was observed in the HP content for all the samples prepared without ascorbic acid, consistent with our Western blot analysis, which showed equal levels of collagen type I in all samples (Fig. 1c). However, in the presence of ascorbate, the differences in HP content measured between nontransfected and stably transfected cells were significant. The HP concentration reported, ΔHP, is the difference in HP between samples grown in the presence and absence of ascorbic acid and is estimated to be the HP present in the ECM. After 1 week, samples overexpressing DDR1/ECD (or DDR2/ECD) exhibited only 18% (or 3%) of the HP content of

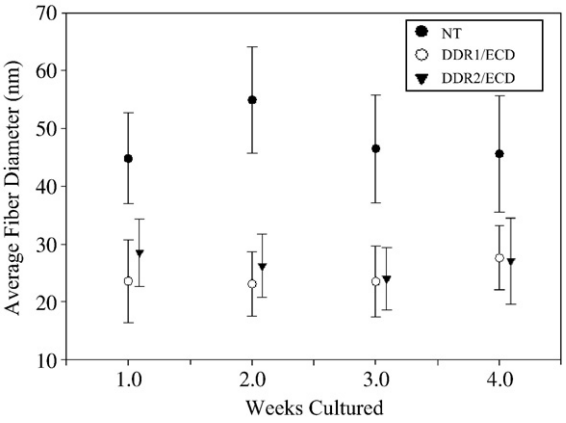

Fig. 4. Overexpression of soluble DDR1/ECD and DDR2/ECD in the ECM reduces the lateral growth of collagen fibers. All cell lines had little to no increase in fiber diameter throughout the 4-week period. The nontransfected cell line maintained an average fiber diameter 40% to 60% higher than those of DDR1/ECD and DDR2/ECD cell lines at all time intervals. Analysis was conducted for 200 fibers for each sample type at every time interval.

nontransfected cells. After 2 weeks, only 52% (or 57%) of the HP content present in nontransfected cell samples was observed for our stably transfected cells. Since HP concentration is indicative of collagen abundance,[25] our results show that collagen deposition in the ECM was significantly reduced in DDR/ECD cell samples.

DDR ECDs promote matrix mineralization

The osteoblastic cell line MC3T3-E1 has previously been utilized to study bone matrix formation including mineralized plaques.[21,26] In order to ascertain how DDR ECDs alter the formation of mineralized calcium deposits *in vitro*, a modified von Kossa staining protocol was employed and visualized with differential interference contrast microscopy. Samples of nontransfected and stably transfected DDR1/ECD, DDR2/ECD, and DDR2/-KD[2] cells were cultured for 2 weeks in the presence of ascorbic acid and in the presence of both ascorbic acid (25 μg/ml) and the osteogenic supplement 2 mM β-glycerophosphate. As seen in Fig. 7, all nontransfected cells showed heterogeneity in crystal size and distribution of crystal formation throughout the sample. The nontransfected cells in the presence of β-glycerophosphate exhibited increased crystal sizes compared to those without the osteogenic supplement. All stably transfected cell lines exhibited a striking increase and a wider distribution of crystal sizes as compared to nontransfected cells even in the absence of β-glycerophosphate. In the presence of

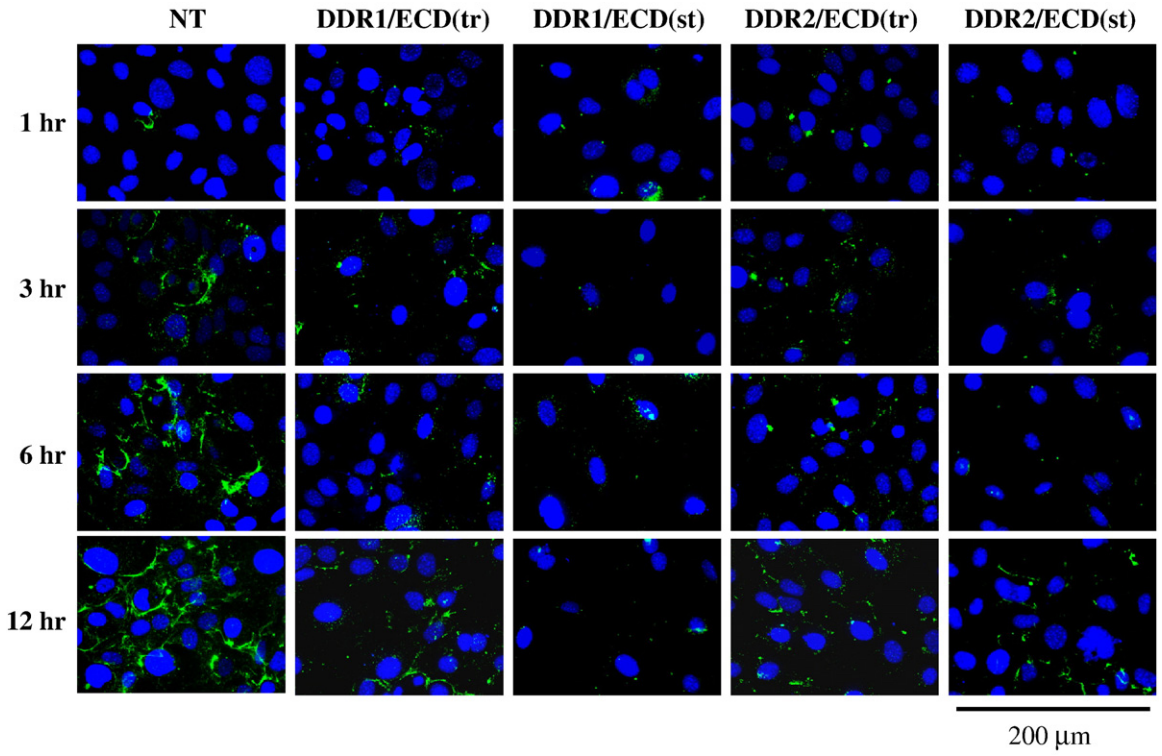

Fig. 5. DDR1/ECD and DDR2/ECD impedes exogenous collagen fibrillogenesis. FITC-labeled collagen was added to native and DDR ECD transiently and stably transfected cell cultures and incubated for 1-, 3-, 6-, and 12-h intervals. Growth of polymeric collagen fiber formation is observed from 1 h through the 12-h period for the native cells (NT). Some fiber formation occurs at the 6- and 12-h time intervals for the transiently transfected samples, DDR1/ECD(tr) and DDR2/ECD(tr) cell cultures, with little to no collagen fiber formation occurring in the stably transfected samples, DDR1/ECD(st) and DDR2/ECD(st). Images were collected using fluorescent microscopy at 63× magnification.

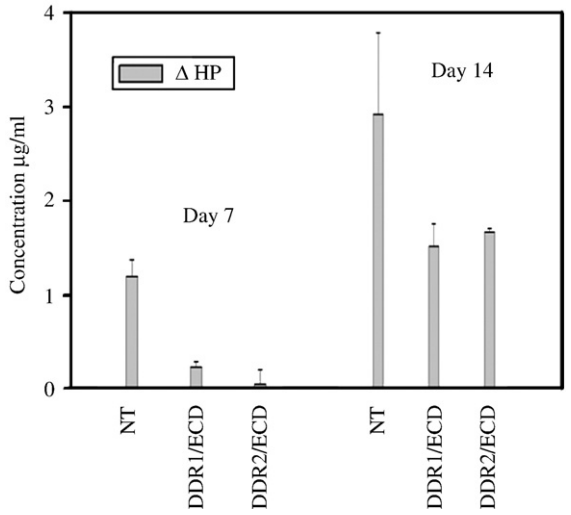

Fig. 6. The abundance of HP present in the ECM of nontransfected (NT) cells is greater than that of DDR ECD transfected cells indicative of collagen content. ΔHP is the difference in HP content between samples grown with and without ascorbic acid. In the absence of ascorbic acid, there is negligible difference in the quantity of HP present across all samples (data not shown). Differences in ΔHP demonstrate collagen content is reduced in cell lines overexpressing DDR1/ECD and DDR2/ECD. Nontransfected cells clearly displayed higher HP content after both 7 and 14 days of culture.

β-glycerophosphate, the DDR2/ECD cells displayed especially pronounced crystal sizes compared to those of all other samples.

Discussion

In this study, we elucidate how cell-secreted, soluble ECDs of DDR1 and DDR2 inhibit collagen fibrillogenesis and enhance matrix mineralization for ECM endogenously generated by the cells. Using similar experimental approaches, in our earlier work[2] we had demonstrated that kinase-deficient and membrane-anchored DDR2 ECD (DDR2/-KD) also inhibits collagen fibrillogenesis. Such an inhibition of collagen fibrillogenesis by DDR1[18] and DDR2[19] ECD was originally observed by us using purified proteins *in vitro*. Thus, our current investigations along with our previous studies[2,18,19] enable us to compare the role of membrane-bound *versus* soluble proteins (DDR-ECD) in regulating collagen fibrillogenesis.

Multiple protein species are known to naturally exist for the transmembrane receptors DDR1 and DDR2. Five splice variants have been characterized for DDR1 ("a" through "e").[12] The d and e isoforms lack the intracellular kinase domain of DDR1. The splicing of DDR1 to various extents has been reported in human ovarian cancer,[27] breast cancer,[15] and fetal brain[15] and has thus far been best characterized in colon cancer cell lines[12]. In normal and diseased arteries of nonhuman primates, three isoforms (a, b, and d) have been detected; these isoforms are differently expressed in advanced atherosclerotic lesions[5] and have been detected in normal human lung tissue and cultured human smooth muscle cells (SMCs).[5] Although splice variants for DDR2 have not yet been characterized, there is ample evidence to support they exist. In one report, Northern blot analysis using a cDNA probe corresponding to the ECD of DDR2 has revealed multiple mRNA species in melanoma carcinoma and virus-transformed normal embryonic lung cell

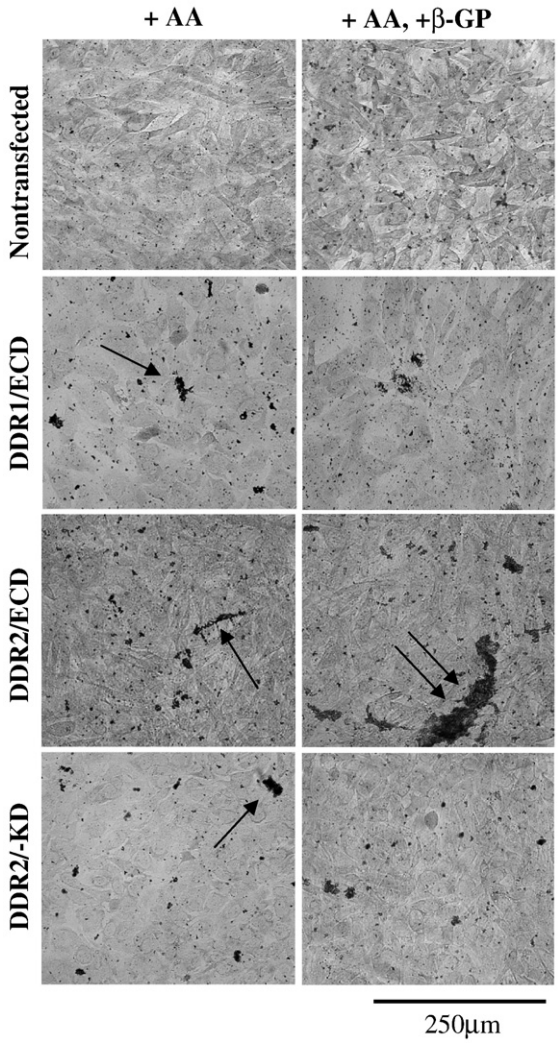

Fig. 7. DDR ECD alters the formation of mineralized plaque in cells. Nontransfected MC3T3-E1 cells display small crystalline calcium deposits (black) throughout the sample; the size of the crystals in these samples increases slightly upon addition of the osteogenic supplement β-glycerophosphate (β-GP) to the culture media. Stable cell lines DDR1/ECD, DDR2/ECD, and DDR2/-KD show increased crystal size as compared to nontransfected cells when cultured in the presence of ascorbic acid (AA), indicated by arrows. Addition of β-glycerophosphate (β-GP) further increases the size of calcium deposits in stable cell lines DDR2/ECD and DDR2/-KD as compared to nontransfected cells. The DDR2/ECD cell line shows the most pronounced calcified crystals, indicated by double arrows. All samples were cultured for 2 weeks.

lines.[15] In a separate study, a major 10-kb transcript and a minor 4.5-kb transcript for DDR2 were detected in human and rat heart and other tissues using Northern analysis,[16] along with additional weak bands at 0.8, 3.6, 2.4, and 1.7 kb. In mouse and rat heart tissue, a DDR2 probe hybridized to multiple RNAs of varying lengths (~4 and 7 kb).[17] At least two transcripts for DDR2 (9.5 and 4.5 kb) and several protein species (130, 90, 50, and 45 kDa) have been found in cultured human SMCs using antibodies against DDR2 ECD.[5] Besides alternate splicing of DDRs, shedding of the DDR1 ECD as a soluble protein in the ECM is another naturally occurring phenomenon reported for several mammalian cells.[13,14] While no direct evidence for DDR2 ECD shedding exists, the Western blots with DDR2 antibodies on SMCs transiently transfected with full-length DDR2 show several smaller molecular species (90, 50, and 45 kDa) besides the full-length 130-kDa receptor, suggesting the likelihood of shedding of DDR2 ECD. Our current investigations highlight the relevance of further characterizing DDR2 isoforms and understanding the different functional roles of DDR1 and DDR2 protein variants.

Several reports by us and others have highlighted that dimerization or oligomerization of DDR1 ECD and DDR2 ECD enhances their binding to triple-helical collagen.[18,19,22,28–31] The ECD of DDRs consists of a discoidin domain and a stalk region. It has been reported that independent deletion of the cytoplasmic domain of DDR1 did not inhibit receptor dimerization.[28] More recently, Abdulhussein *et al*. found that the cysteine residues, 303 and 348 present in stalk region of DDR1 ECD are essential for receptor dimerization and deletion of the stalk region prevented receptor dimerization.[30] Further, Abdulhussein *et al.* demonstrated that glutathione *S*-transferase (GST) tagged DDR1 ECD and DDR2 ECD when expressed as a soluble protein bound to collagen.[29] The molecular mass of the DDR1 ECD-GST and DDR2 ECD-GST proteins was found to be around 62 kDa,[29] similar to the molecular masses of the DDR1/ECD and DDR2/ECD proteins utilized in this study. We have also recently shown that DDR1 exists as a dimer on the cell surface, independent of the presence of collagen, and undergoes further oligomerization upon ligand stimulation.[31] Since the DDR1/ECD and DDR2/ECD proteins in our stable cell lines preserve the capacity to interact with collagen, they are likely expressed as dimers and may undergo further oligomerization upon interaction with collagen.

Our results signify that both membrane-anchored and soluble isoforms of DDR ECD proteins may be important in ECM remodeling. In this regard, several soluble collagen-binding proteins secreted in the ECM—decorin,[3,32–34] lumincan,[3,34,35] biglycan,[34,36] fibromodulin,[34–36] periostin,[37] aggrecan,[34] and versican[34]—are known to regulate collagen fibrillogenesis. Decorin and lumican are known to regulate collagen fibril diameter,[3,38] and the absence of biglycan and fibromodulin inhibits the maturation of collagen fibrils.[36] Animal studies have begun to reveal the importance of soluble collagen-binding proteins in the regulation of collagen maturation and fiber diameter. Studies on knockout mice for decorin,[39] lumican,[35] fibromodulin,[35,36] and periostin[37] have demonstrated that these proteins are critical to generate a uniform collagen fiber diameter in tissues. For example, ultrastructural analyses of the cornea, skin, and tendon from lumican knockout mice shows collagen fibers with increased fiber diameters, while the tendon from fibromodulin knockout mice contains higher frequency of smaller fiber diameters.[35] Limited studies exist on the effects of membrane-anchored proteins on fibrillogenesis of type 1 collagen. Integrins $\alpha_5\beta_1$ and $\alpha_2\beta_1$ are understood to modulate collagen fibrillogenesis predominantly with fibronectin polymerization as a prerequisite.[40] Although the integrin α_1(I) and α_2 (I) domains have been shown to affect collagen fibrillogenesis as soluble proteins *in vitro*,[41] no changes in collagen fiber density or organization were observed in the integrin $\alpha_1\beta_1$[42] or α_2 subunit-deficient mice.[43] Interestingly, the knockout mice for the orphan receptor Gpr48[44] and the transmembrane collagen XIII[45] showed disrupted collagen fibrils, although the mechanisms of their interaction with collagen type 1 are not well understood. Our current results along with our previous findings indicate that DDR ECDs serve as a robust model system to compare and contrast how membrane-anchored *versus* soluble proteins may regulate collagen fiber structure and deposition.

We had earlier reported that collagen fibers with intact native banded structure were occasionally observed in the kinase-deficient, membrane-anchored DDR2 ECD (DDR2/-KD) samples; however, in our DDR1/ECD and DDR2/ECD samples, observation of native banded structure of collagen was far more infrequent. D-periodicity of collagen fibers from native cultures was measured at 61 ± 5 nm, which is in agreement with previous studies by us and others.[46] Previously, we found that the membrane-anchored DDR2/-KD inhibited lateral fiber growth, compared to native cultures. While fiber diameter measurements for the first week of culture for DDR1/ECD and DDR2/ECD gave results similar to those of DDR2/-KD samples (25.0–28.9 nm), fibers in DDR2/-KD cultures exhibited lateral growth of around 10 nm over the 3 weeks of culture observed. In contrast, a sustained inhibition of lateral growth of collagen fibers was observed by DDR ECD proteins resulting in average collagen fiber diameters between 20 and 30 nm throughout a 4-week period. Together, our results show that soluble DDR2 ECD inhibits collagen fibrillogenesis in the ECM consistent with membrane-anchored DDR2, albeit with a slightly higher potential. We speculate that this stronger inhibition of collagen fiber structure and lateral diameter is due to the soluble DDR ECD being distributed throughout the ECM and thus having more ability to affect collagen fiber formation even in ECM regions away from the pericellular regions.

We found that both DDR1 and DDR2 ECD increased matrix mineralization as compared to native cells, with the effect of DDR2 ECD being more prominent. Both soluble (DDR2/ECD) and membrane-bound DDR2 ECD (DDR2/-KD), when compared to wild-type cells, induced larger mineral deposits. In this regard, a recent study has reported abnormal calcification arising due to mutations in the DDR2 gene in spondylo-meta-epiphyseal dysplasia (SMED) in humans.[47] It is interesting to note that all the mutations reported were found in the DDR2 intracellular domain and not in its ECD. Although the expression levels of DDR2 were not reported in this study,[47] it is likely that expression of DDR2 ECD present in the full-length mutated receptor in SMED cases along with impaired signaling of the mutated receptor may lead to increased calcification. Matrix mineralization in both DDR1 and DDR2 knockout mice have not been reported in detail; however, in DDR1 knockout mice, reduced bone calcification was described in the fibula bone.[48] Our observations suggest the importance of evaluating matrix mineralization with respect to expression of both the full-length DDR receptors and their isoforms containing the ECDs. Since the collagen type I binding site for decorin is in close proximity to that of DDR2,[49] further investigations are needed to understand if binding of DDR2 ECD to collagen type I promotes crystal formation by interfering with decorin binding. It is interesting to compare the effect of DDRs on collagen fibrillogenesis and matrix mineralization to those of decorin. Both DDR ECDs and decorin inhibit collagen fibrillogenesis and result in reduction of collagen fiber diameters.[33] In contrast, while DDR ECDs enhance matrix mineralization, decorin is found to be an inhibitor of collagen calcification.[4] No reports elucidating the ultrastructure of native ECM in DDR1 or DDR2 knockout mice have yet been made.

We conclude that expression of both membrane-bound and soluble DDR1 and DDR2 ECDs can alter the morphology of endogenous collagen fibers, thus perturbing the overall ECM structure. We speculate that such perturbations, if observed *in vivo*, may significantly alter the integrity and biomechanical properties of resulting tissues. Further studies need to be addressed to elucidate which DDR1 and DDR2 isoforms are modulated in pathological states *in vivo* and how their expression alters ECM morphology and tissue biomechanics.

Materials and Methods

Creation of expression constructs for soluble DDR ECD

Expression plasmids encoding the ECD of DDR1 and DDR2 were generated using the full-length mouse DDR1-myc and DDR2-myc constructs obtained from Regeneron Pharmaceuticals, Tarrytown, NY.[50] The coding regions of the DDR1/ECD (amino acids Met1 to Ser412) and DDR2/ECD (amino acids Met1 through Ile400) were amplified by PCR utilizing the Pfu TURBO polymerase (Stratagene, La Jolla, CA) and the following primers:

DDR1/ECD:
Forward: 5′-GAAGGATGGGGACAGGGACC-CTC-3′
Reverse: 5′-GCTCCCCTCCGCCTTGCCCAC-3′
DDR2/ECD:
Forward: 5′-AGGATGATCCCGATTCCCAGA-3′
Reverse: 5′-GATCCGAGTGTTGCTATCATC-AAC-3′

The resulting PCR products (DDR1/ECD, 1241 bp; DDR2/ECD, 1203 bp) were subjected to *Taq* polymerase to include 3′ A-overhangs in the PCR product to enable ligation immediately into the pcDNA3.1/V5-His-TOPO vector using the Top10 chemically competent cells from Invitrogen. Recombinant clones were identified by restriction analysis using double digestion with Age1 and Kpn1 (for DDR1/ECD) and Kpn1 and EcoRV (for DDR2/ECD). The authenticity (i.e., correct orientation and in-frame with the V5 coding region) of the resulting clones was verified by dideoxynucleotide sequencing. Creation of expression plasmid encoding for the DDR2/-KD construct was reported earlier.[2]

Creation of stable cell lines

The pre-osteoblast mouse cell line MC3T3-E1 (ATCC) has been used extensively to elucidate functional roles of osteoblastic cells and collagen fibrillogenesis.[21,26] Cells were seeded (60–80% confluent) on two 100-mm dishes in MEM-α with 10% (v/v) fetal bovine serum and 1% penicillin–streptomycin (Gibco). Cells were transfected with DDR1/ECD and DDR2/ECD expression construct using FuGene 6 (Roche). Thirty hours after transfection, the cells were incubated with selection media containing geneticin (475 μg/ml). After 12 to 14 days of culture in selection media, surviving cells from DDR1/ECD and DDR2/ECD colonies were transferred to 35-mm dishes. Of the many stable cell lines generated, two were selected. Selection was done on the basis of healthy cell morphology and expression level of the DDR ECD proteins. Western blotting was performed on whole-cell lysates or conditioned media from serum-starved cultures for verification of protein expression. One DDR1/ECD and one DDR2/ECD stable colony was selected based on protein expression and healthy cell morphology.

Transmission electron microscopy

Nontransfected MC3T3-E1 and stably transfected DDR1/ECD and DDR2/ECD samples were prepared for TEM as previously reported.[2] Briefly, cells were cultured in the presence of ascorbic acid (25 μg/ml) for 1 to 4 weeks. Ascorbic acid is critical for prolyl hydroxylation and for lysine hydroxylase activity in the biosynthesis and assembly of collagen.[51] Samples were fixed in 4% (v/v) gluteraldehyde and stained with 1% osmium tetroxide and uranyl acetate. Dehydration was carried out by a graded ethanol series (30–100%) and samples were embedded in an epoxy resin. Thick sections (80 nm) were obtained by use of a Leica Ultracut UCT ultramicrotome (Leica-Micro-systems, Vienna, Austria). Sections were examined with a Zeiss EM 900 transmission electron microscope (Carl-Zeiss SMT, Peabody, NY) operating at

80 kV. An Olympus SIS Megaview III camera (Lakewood, CO), was used to obtain digital micrographs at magnifications ranging from 7000× to 85,000×.

TEM image analysis

Image J software (NIH) was used on TEM images to measure the diameter of collagen fibers present in the ECM. Fiber diameters were measured on cross-sectional or longitudinal images of collagen fibers. TEM micrographs with magnifications of 50,000× and 85,000× were utilized. For each specimen type, at least three independent cell cultures were made and processed. At least three TEM grids were made from each sample, and numerous regions on each grid were imaged. Two hundred fiber diameters were measured for each specimen type, and a statistical analysis provided average diameter, standard deviation, and frequency distribution data. The morphology of collagen fibers was qualitatively analyzed in TEM micrographs to identify the presence or absence of D-periodicity in collagen fibers.

HP assay

HP assays were performed on nontransfected MC3T3-E1, DDR1/ECD, and DDR2/ECD stably transfected cell cultures to determine the collagen content in the adherent cell layer as previously described.[2,25] Briefly, cell cultures were prepared in triplicate, with and without ascorbic acid, and cultured for 7 and 14 days. A cell proliferation assay was used to normalize the cell population of each sample using Calcein-Am (BioChemika 17783) in order to reduce discrepancies due to variation in growth rate of the samples. The adherent cell layers were scraped and pipetted into individual Eppendorf tubes and brought to a volume of 50 μl with a final concentration of 4 N sodium hydroxide and autoclaved for 20 min at 120 °C. Chloromine T reagent (450 μl) was added to each sample and samples were incubated at room temperature for 25 min. Lastly, 500 μl of Ehrlich's reagent was added to each sample and samples were incubated at 65 °C for 20 min. Absorbance was measured at 560 nm with a Beckman DU730 spectrophotometer. The HP content was obtained by calibration against a standard curve ranging from 0.5 to 10 μg/ml. For each cell line the difference in HP content, ΔHP, between cells grown in the presence and absence of ascorbic acid was calculated. ΔHP is thus indicative of the amount of collagen present in the ECM.

Fluorescence microscopy

Fluorescence microscopy was used to ascertain the rate of collagen fibrillogenesis by nontransfected MC3T3-E1 cells and cells stably and transiently transfected with DDR1/ECD and DDR2/ECD for exogenously added fluorescently labeled collagen. Cells were cultured on 1% (w/v) poly-L-lysine-coated glass coverslips and grown in the absence of ascorbic acid in order to ensure that no endogenous collagen fibrillogenesis by the cells occurs in the assay. Cells were incubated with FITC-conjugated collagen type 1 (Sigma C4361) at a final concentration of 1 μg/ml for the appropriate time intervals: 30 min, 1, 3, 6, and 12 h. Cells were washed and fixed with 2% formalin (Fisher Scientific, Kalamazoo, MI) for 30 min. Thereafter, cells were washed and incubated for 20 min with 4′,6-diamidino-2-phenylindole (DAPI) for nuclear staining. Glass coverslips were mounted onto microscope slides using ProLong Gold antifade reagent (Invitrogen Molecular Probes P36934). Slides were examined with a 63× objective on a Zeiss Axiovert 200 microscope. An EXFO mercury lamp was used to excite the sample, and appropriate filter cubes were used for fluorochrome observation: YFP-2427A (Semrock) was used to observe FITC and filter set # 49 (Zeiss) was used for observance of DAPI nuclear stain.

Mineralization assay

Nontransfected MC3T3-E1 cells and stably transfected DDR1/ECD, DDR2/ECD, and DDR2/-KD (previously published[2]) cells were seeded onto 25-mm glass coverslips. For each cell type, samples were grown in duplicate either in the presence of ascorbic acid (25 μg/ml) or in the presence of both ascorbic acid (25 μg/ml) and 2 mM β-glycerophosphate (an osteogenic supplement). After 14 days of culture, cells were fixed in 4% paraformaldehyde for 15 min at room temperature and washed three times in ultrapure water. To visualize calcium deposits, a modified von Kossa staining procedure was employed.[26] $AgNO_3$ [2.5% (w/v)] was placed on the cells and cells were allowed to incubate for 30 min in the dark after which cells were exposed to UV light for 50 min. Cultures were washed three times in ultrapure water, counterstained with toluidine blue for 3 min, washed repeatedly, and finally mounted onto microscope slides. Micrographs were imaged using differential interference contrast with a 20× objective lens on a Zeiss Axiovert 200 microscope. The experiment was repeated three independent times.

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