Partial characterization of cell–type X collagen interactions

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Type X collagen is a short-chain non-fibrillar collagen that is deposited exclusively at sites of new bone formation. Although this collagen has been implicated in chondrocyte hypertrophy and endochondral ossification, its precise function remains unclear. One possible function could be to regulate the processes of chondrocyte hypertrophy through direct cell–type X collagen interactions. Adhesions of embryonic chick chondrocytes, and cell lines with known expression of collagen-binding integrins (MG63 and HOS), were assayed on chick type X collagen substrates, including the native, heat-denatured and pepsin-digested collagen, and the isolated C-terminal non-collagenous (NC1) domain. Type X collagen supported the greatest level of adhesion for all cell types tested. The involvement of the $\alpha2\beta1$ integrin in type X collagen–cell interaction was demonstrated by adhesion studies in the presence of Mg^{2+} and Ca^{2+} ions and integrin-function-blocking antibodies. Cells expressing $\alpha2\beta1$ integrin (chick chondrocytes and MG63 cells) also adhered to heat-denatured type X collagen and the isolated NC1 domain; however, removal of the non-collagenous domains by limited pepsinization of type X collagen resulted in very low levels of adhesion. Both focal contacts and actin stress-fibre formation were apparent in cells plated on type X collagen. The presence of $\alpha2$ and $\beta1$ integrin subunits in isolated chondrocytes and epiphyseal cartilage was also confirmed by immunolocalization. Our results demonstrate, for the first time, that type X collagen is capable of interacting directly with chondrocytes and other cells, primarily via $\alpha2\beta1$ integrin. These findings are atypical from the fibrillar collagen–cell interactions via collagen binding integrins in that: (1) the triple-helical conformation is not strictly required for cell adhesion; (2) the NC1 domain is also involved in the adhesion of $\alpha2\beta1$-expressing cells. These data form the basis for further studies into the mechanism and biological significance of type X collagen deposition in the growth plate.

Key words: adhesion, chondrocyte, endochondral ossification, integrin, NC1 (C-terminal non-collagenous) domain, type X collagen.

INTRODUCTION

Longitudinal bone growth by endochondral ossification (EO) occurs in the epiphyseal growth plate where chondrocytes proliferate, mature and undergo hypertrophy {accompanied by changes in the extracellular matrix (ECM) that is synthesized [1]}, followed by cartilage mineralization and subsequent bone formation. The process of EO is also important in bone repair and pathology (such as in fracture callus development and ectopic bone formation in osteoarthritis [2,3]). Numerous factors which regulate the process of EO have been identified, including insulin-like growth factors, transforming growth factor-β, fibroblast growth factors and the Indian Hedgehog (Ihh)/parathyroid-hormone-related-protein feedback loop [4,5].

ECM components are known to regulate numerous cellular activities, either by direct cell–matrix interactions or by facilitating growth-factor binding [6]. Indeed, integrin-mediated cell–matrix interactions are critical to many of these processes [7–9]. Furthermore, anti-integrin antibodies have been shown to inhibit chondrocyte terminal differentiation, highlighting the importance of cell–matrix interactions in the process of chondrocyte maturation and hence EO [10,11]. It is conceivable that developmentally regulated matrix molecules may have roles besides the maintenance of tissue structure and integrity. Since type X collagen is only deposited in the hypertrophic matrix [12,13], it may possess both structural and regulatory roles in EO.

Type X collagen is a homotrimer of three $\alpha1$(X) chains (molecular mass 59 kDa) comprising a 45 kDa triple-helical domain and two non-collagenous domains: a small N-terminal domain (NC2) and a larger C-terminal one (NC1) [14]. Mutations of the human Col10A1 (the gene for type X collagen $\alpha1$ chain) are known to cause growth-plate compression and associated complications known as metaphyseal chondrodysplasia Type Schmid [15,16], with the majority of mutations being found in the NC1 domain [17]. Studies of vitamin D deficiency rickets [18], avian tibial dyschondroplasia [19] and transgenic mice [20–22] highlight the importance of the type X collagen matrix in chondrocyte hypertrophy, matrix mineralization and vascular invasion [23]. However, the precise role of type X collagen in EO remains unclear. Unlike the cartilage fibrillar collagens, this molecule assembles into a hexagonal lattice [24]. Several roles for type X collagen have been suggested, including the maintenance of tissue stiffness [25]. Our studies of chick tibial dyschondroplasia have shown that failure of chondrocytes to reach full cell hypertrophy may result from the failure of type X collagen deposition [19]. We therefore postulate that pericellular type X collagen might regulate chondrocyte metabolism and the process of EO by interacting with hypertrophic chondrocytes through cell adhesion molecules such as integrins.

METHODS

All tissue-culture reagents were obtained from Gibco Invitrogen Corporation, whereas all other reagents were obtained from Sigma Chemical Co. (unless otherwise stated).

Abbreviations used: ECM, extracellular matrix; NC1 domain, C-terminal non-collagenous domain; EO, endochondral ossification; DMEM, Dulbecco's modified Eagle's medium; FCS, foetal-calf serum; nX, native type X collagen; *GAPDH*, gene coding for glyceraldehyde-3-phosphate dehydrogenase; HI-BSA, heat-inactivated BSA; Ihh, Indian Hedgehog; PBS–, Ca^{2+}- and Mg^{2+}-free PBS; RT-PCR, reverse-transcription PCR.

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Preparation of cells

Embryonic-chick chondrocytes were isolated from tibial epiphyses of 17-day-old White Leghorn chick embryos. The prehypertrophic and hypertrophic zones were separated by dissection at the border of the hypertrophic zone under a dissecting microscope. Cells were maintained in Dulbecco's modified Eagle's medium (DMEM) containing 10 % (v/v) heat-inactivated foetal-calf serum (FCS) (DMEMFCS10) as previously described [26]. The homogeneity of the cell populations obtained was examined by reverse-transcription (RT-) PCR as described below. Cell morphology was also examined by phase-contrast microscopy. Briefly, freshly isolated chondrocytes were seeded on to eight-well chamber slides (Lab-Tek II; Nalge Nunc International, Rochester, NY, U.S.A.), grown overnight and representative photomicrographs taken. The human osteosarcoma cell lines MG63 and HOS were also examined because of their defined expression patterns of collagen-binding integrins, and were obtained from the European Collection of Animal Cell Cultures (ECACC) (Centre for Applied Microbiology and Research, Porton Down, Salisbury, Wilts., U.K.). MG63 and HOS cells were maintained as monolayer cultures in DMEMFCS10.

Preparation of chick type X collagen

Native chicken type X collagen (nX) was isolated from chick hypertrophic chondrocyte conditioned media according to the methods of Barber and Kwan [26]. In order to gain insights into the nature and location of cell-binding site(s) within type X collagen, pepsinized type X (i.e. type X collagen lacking the non-collagenous domains) and heat-denatured type X collagen [i.e. non-triple helical $\alpha 1$(X) chains] were also generated, as well as the isolated NC1 domain. Pepsinized type X collagen was prepared by incubation with 100 μg/ml pepsin (overnight, 4 °C), heat-denatured type X by boiling for 15 min, and the isolated NC1 domain as previously described [26].

RNA isolation and RT

Embryonic-chick hypertrophic and prehypertrophic chondrocytes were isolated and grown overnight as described above. After a brief PBS wash to remove non-adherent cells, the cells were resuspended by collagenase/trypsin digestion and RNA extracted using TRI-reagent (Sigma) at 5×10^5 cells/ml (following the manufacturer's instructions). The isolated chick RNA was treated with RQ1 RNase-free DNase (Promega) to remove contaminating genomic DNA at 37 °C for 30 min. The resultant RNA preparation was further purified using the RNeasy Mini kit (Qiagen, Crawley, Sussex, U.K.) as described by the manufacturer. A 1 μg portion of purified RNA was reverse-transcribed to cDNA using murine-Moloney-leukaemia-virus reverse transcriptase and random hexamers (Promega) at 42 °C for 60 min before PCR.

PCR

In order to confirm the identity and homogeneity of the embryonic chick chondrocyte preparations obtained, PCR was carried out using primers for a selection of markers of chondrocyte differentiation, namely types II and X collagen, Ihh, the anti-apoptotic protein Bcl-2, as well as housekeeping genes *GAPDH* (coding for glyceraldehyde-3-phosphate dehydrogenase) and c-*myc* (Table 1). PCR reactions were carried out using 50 μl reaction volumes. Each reaction mixture contained $1 \times$ *Taq* polymerase buffer (Promega), 150 ng of each primer, 2 units of *Taq* polymerase (Promega), 1.5 mM $MgCl_2$, 1 μl of RT product, and 1 μl of 10 mM dATP, dGTP, dCTP and dTTP (Promega). Samples were heated to 94 °C for 30 s in a PerkinElmer GeneAmp 9700 thermocycler prior to PCR. The PCR conditions were as follows: 35 cycles of [94 °C (30 s), annealing temperature (see Table 1) (1 min), and 72 °C (30 s), followed by 5 min at 72 °C. The resultant PCR products were electrophoresed on 1.5 % agarose gels.

Table 1 Primers for a selection of markers of chondrocyte differentiation

Primer	Primer sequences	Annealing temperature (°C)
Chick Type II collagen	Forward 5′-CCAACACCGCCAGCATCC Reverse 5′-GCCAATATCCACGCCAAACTCCT	56
Chick Type X collagen	Forward 5′-AGCAGGAGCAAATCAAGC Reverse 5′-CAGAGGAATAGAGACCAT	52
Chick Ihh	Forward 5′-CATCATCTTCAAGGACGAGGAGAAC Reverse 5′-TACTTGTTGCGGTCCCTGTCTGAC	40
Chick Bcl-2	Forward 5′-ATACTGACAAAGACCTACCTG Reverse 5′-GCGATGGCACGACTGATAACT	52
Chick *GAPDH*	Forward 5′-AAGGCTGAGAACGGGAAACTTG Reverse 5′-TCAACAACAGAGACATTGGGGG	56
Chick c-*myc*	Forward 5′-CACAGAGTCCAGCACAGAAGCATC Reverse 5′-TGGTCACGCAGGGCAAAGAAAC	56

Cell-adhesion assays

Plates (96-well; ICN Pharmaceuticals Ltd., Basingstoke, U.K.) were coated (4 °C, 18 h) with 1–20 μg/well intact purified nX; 1–10 μg/well purified native NC1 domain (nNC1), 10 μg/well heat-denatured NC1, pepsinized type X collagen, heat-denatured pepsinized type X collagen, heat-denatured type X collagen, rat tail tendon Type I collagen or bovine nasal cartilage Type II collagen. Positive control wells were coated with 1 mg/well concanavalin A in PBS for 2 h at 37 °C. Negative control wells were coated with 100 μl of 10 mg/ml heat-inactivated BSA (HI-BSA) in PBS (prepared by heating to 90 °C for 30 min).

After trypsinization, cells were resuspended in either DMEM, DMEM containing 10 mM calcium or DMEM containing 10 mM magnesium, at 5×10^4 cells/ml. Plates were blocked (30 min, 37 °C) with HI-BSA in PBS, washed with Ca^{2+}- and Mg^{2+}-free PBS (PBS–) and 100 μl/well cell suspension added. Plates were incubated for 3 h, washed with PBS–, and the number of adherent cells and percentage cell spreading assessed by optical microscopy and cell counting. Cell adhesion to the various substrates was then expressed as a percentage of adhesion to concanavalin A. Cell adhesion to concanavalin A was used as a positive adhesion control, since it supports the adhesion of greater than 90 % of the cell suspension added. Cell adhesion to uncoated blocked plastic (negative control) did not normally exceed 1 %. Cells that were no longer exhibiting a round-cell morphology and beginning to acquire a polygonal shape were counted as spread cells.

Cell adhesion to type X collagen was also assessed in the presence of 1 μg/well of function-blocking monoclonal antibodies against chick $\beta 1$-integrin subunit (W101B; Sigma), human $\beta 1$-integrin subunit (Mab13; kindly donated by Professor

Martin Humphries, School of Biological Sciences, University of Manchester, Manchester, U.K.) and human $\alpha2\beta1$ (BHA2.1; Chemicon International, Harrow, Middx., U.K.) and 10–20 μg/well purified NC1 domain. All statistical analyses were carried out by two-sample Student's *t* test.

Localization of integrin subunits and focal contacts

For the immunolocalization of $\alpha2$ and $\beta1$ integrin subunits, 10 μm cryosections of 17-day-old embryonic-chick tibial epiphyses were fixed in 95% (v/v) ethanol for 30 s and air-dried. Chondrocytes (cultured overnight) were fixed [95% (v/v) ethanol, 30 s], air-dried and permeabilized by the addition of 0.1% (v/v) Triton X-100 for 15 min. Cryosections were then blocked for 20 min with 1:20 goat serum in PBS containing 0.1% (v/v) Tween-20 (PBS-T). Primary antibody (1:2 dilution of culture supernatant) was then added for 1 h. The anti-(chick $\beta1$ subunit) monoclonal antibody V2E9 was obtained as hybridoma culture supernatant from the Developmental Studies Hybridoma Bank (Department of Biological Sciences, University of Iowa, Iowa City, IA, U.S.A.). Culture supernatant containing the anti-($\alpha2$ subunit) antibody MEP-17 was a gift from Dr Kelly McNagny, European Molecular Biology Laboratory, Heidelberg, Germany. Specimens were then washed with PBS and the appropriate FITC-conjugated antibody (1:50 dilution) added for 1 h. Samples were mounted using Vectashield® (Vector Laboratories, Orton Southgate, Peterborough, U.K.) containing propidium iodide. Mouse or rabbit immunoglobulins were applied to control tissue sections and cell layers (1:25 dilution).

For localization of focal contacts, Lab-Tek II eight-well chamber slides were coated with type X collagen (30 μg/well; overnight; 4 °C), washed in PBS, and blocked with HI-BSA at 37 °C. Hypertrophic chondrocytes were seeded at 3×10^4 cells/well, incubated at 37 °C for 3 h as previously described, and fixed in 70% (v/v) ethanol. Subsequent steps utilised PBS-T in all washes and as antibody diluent. Cells were washed and blocked for 30 min with 1:20-diluted normal goat sera. Slides were then incubated with primary antibody (mouse anti-vinculin; monoclonal; hVin1; 1:200; overnight; 4 °C), and washed with PBS-T. Primary and normal mouse sera controls were carried out in parallel. FITC-conjugated goat anti-mouse secondary antibody, diluted 1:50, was then applied for 1 h. Actin filaments were stained with FITC-conjugated phalloidin (2 μg/ml for 30 min). Slides were then mounted in Vectashield containing propidium iodide, and examined using a Leitz DMRD fluorescence microscope (Leica Microsystems GmbH, Mannheim, Germany).

RESULTS

Chondrocyte populations used for cell-adhesion studies

Optical microscopy and RT-PCR were used to examine the purity of the chondrocytes isolated from 17-day-old embryonic-chick tibia. The two cell populations obtained displayed very different morphologies. Chondrocytes isolated from the prehypertrophic zone of the epiphysis remained small and rounded when cultured on a tissue-culture plastic substratum, a typical morphology of non-differentiating growth-plate chondrocytes in culture. Chondrocytes isolated from the hypertrophic zone were flattened and much larger, which are typical of hypertrophic chondrocytes in culture (Figure 1A). A number of cultures were examined, and no evidence of cross-contamination was observed on the basis of morphological assessments.

RT-PCR of various markers of chondrocyte differentiation was used to assess the homogeneity of the cell populations. Types II and X collagen mRNA were observed to be expressed by both cell populations (Figure 1B, lanes 2 + 3 and 4 + 5 respectively). Expression of Type II collagen mRNA by both cell populations was expected, and indicated the chondrocytic phenotype of the cells in culture. It should be noted that, whereas type X collagen protein is only deposited in the hypertrophic zone, the type X mRNA is detectable in the prehypertrophic zone, indicating that the expression of the type X collagen gene begins at the transition from proliferative to hypertrophic cells [23]. Ihh expression has been shown to be expressed only by prehypertrophic chondrocytes [4]. In our isolated cell populations, Ihh expression was primarily observed in the prehypertrophic cell population (Figure 1B, lane 7). Isolated prehypertrophic chondrocytes also expressed mRNA for BCL-2, whereas hypertrophic chondrocytes (the normal fate of which is to undergo apoptosis [27–29]) showed no expression (Figure 1B, lanes 8 and 9). Down-regulation of BCL-2 in hypertrophic chondrocytes has previously been reported [30]. Expression of the housekeeping genes *GAPDH* and c-*myc* did not vary between cell populations (Figure 1B, lanes 10 + 11 and 12 + 13 respectively). Cell morphology and RT-PCR results, therefore, indicate that the cell populations obtained displayed a high degree of homogeneity.

Adhesion of cells to type X collagen

In order to investigate the ability of type X collagen to support cell adhesion and spreading, a variety of cell types were chosen. Growth-plate chondrocytes were examined, since these cells may directly interact with pericellular type X collagen *in vivo*. The human osteosarcoma cell lines MG63 and HOS were also chosen, since they are known to express distinct collagen-binding integrins [31,32]. nX mediated the adhesion and spreading of all the cell types tested in a concentration-dependent manner (Figures 2A and 2B). Of the cell types tested, hypertrophic chondrocytes exhibited the highest affinity for the type X collagen substrate, whereas HOS cells showed the lowest percentage adhesion to type X (Figures 2A and 2B).

Adhesion and spreading of chick hypertrophic chondrocytes to nX was enhanced by the presence of Mg^{2+} ions and was inhibited by Ca^{2+} ions (Figure 2C) in a manner typical of cell adhesion mediated via integrins [33]. Similar bivalent-cation-dependent adhesion to type X collagen was also observed for a variety of other cell types, including prehypertrophic chick chondrocytes, HT1080 human fibrosarcoma cells and primary human osteoblasts (results not shown). Furthermore, the percentage of chick chondrocytes adhering to type X collagen was significantly decreased in the presence of function-blocking anti-$\beta1$ antibody (Figure 3A). The adhesion of MG63 cells (which express $\alpha2\beta1$ integrin) could also be significantly inhibited by function-blocking anti-$\alpha2\beta1$ and anti-($\beta1$ integrin) antibody (Mab-13), whereas adhesion of HOS cells (which do not express $\alpha2\beta1$ integrin) was not effected by anti-$\alpha2\beta1$ antibody [31,32], but was abrogated by Mab13 (Figure 3B). Taken together, these data strongly suggest that type X collagen possesses adhesive determinants for the $\alpha2\beta1$ integrin. It should, however, be noted that HOS cells, which lack the $\alpha2\beta1$ integrin, but possess the collagen-binding $\alpha1\beta1$ integrin [32], are capable of interaction with type X collagen substrate. It therefore appears that $\alpha1\beta1$ integrin may also be able to support cell adhesion to nX.

It was also observed that the adhesion and spreading of chick chondrocytes (especially hypertrophic cells) to nX was consistently higher than that to types I and II collagens

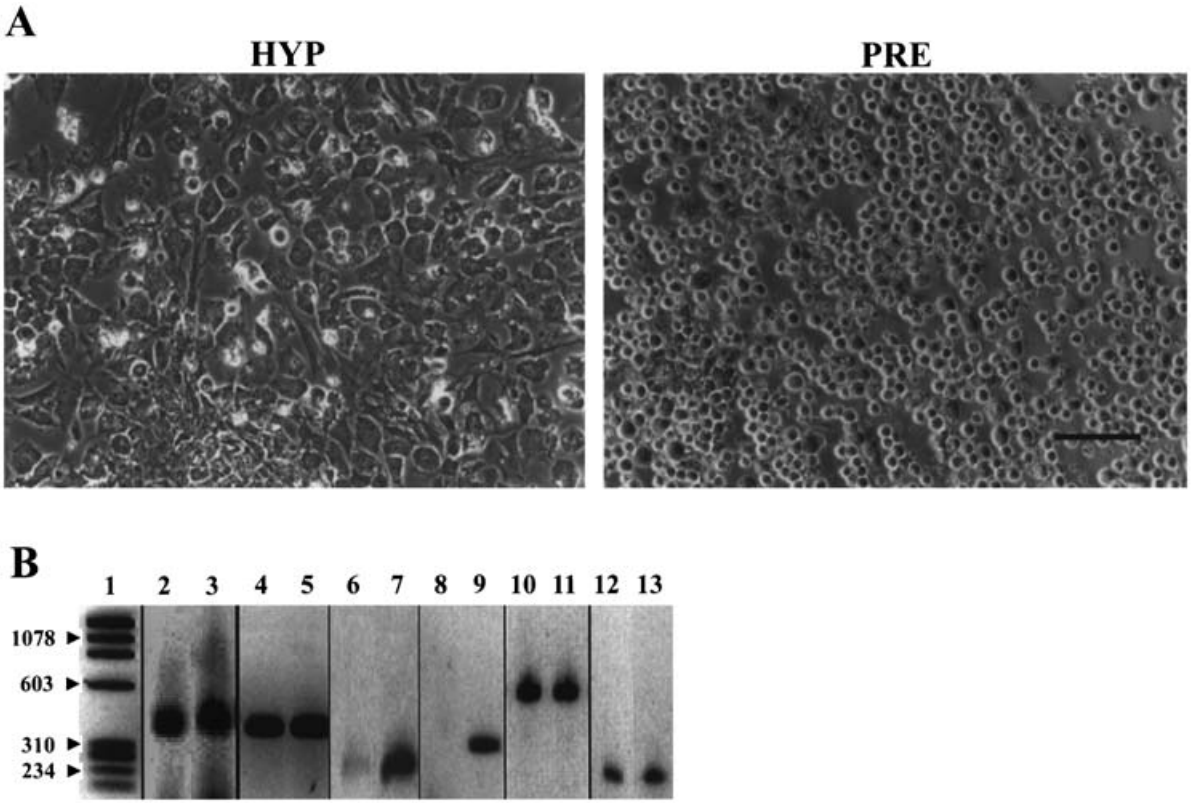

Figure 1 Characterization of the isolated chondrocyte phenotypes by phase-contrast microscopy and RT-PCR

(**A**) Phase-contrast photomicrographs of hypertrophic (HYP) and pre-hypertrophic (PRE) chondrocytes isolated from 17-day-old embryonic-chick tibial epiphyses and cultured for 24 h. The bar represents 50 μm. (**B**) RT-PCR of total RNA isolated from hypertrophic chondrocytes (lanes 2, 4, 6, 8, 10 and 12) and prehypertrophic chondrocytes (lanes 3, 5, 7, 9, 11 and 13). Electrophoretic analyses on 1.5 %-agarose gel of PCR products using primers of: chick Type II collagen (lanes 2 and 3); type X collagen (lanes 4 and 5); Ihh (lanes 6 and 7); Bcl-2 (lanes 8 and 9); *GAPDH* (lanes 10 and 11) and c-*myc* (lanes 12 and 13). Markers (endonuclease-*Hae*III-cleaved fragments of phage ΦX174 DNA) are shown in lane 1.

(Figure 3A). These differences in cell adhesion appear to reflect a higher degree of affinity of chondrocytes for type X collagen, since coating efficiency between the different collagen types did not appear to vary (as assessed by coating plates with the ^{3}H-labelled collagens using methods identical with those described previously, followed by liquid-scintillation counting to assess the amount of unbound collagen remaining in solution; results not shown).

To further elucidate the nature and position of cell-binding sites within type X collagen, pepsinized and heat-denatured type X collagen were generated, along with the isolated non-collagenous NC1 domain. Figure 4 shows that nX supports a greater degree of cell adhesion than pepsinized type X, heat-denatured type X or the isolated NC1 domain. Adhesion of chick chondrocytes to pepsinized type X collagen, which lacks the non-collagenous domains NC1 and NC2, was significantly decreased (from 74 to 11 % for hypertrophic cells and from 41 to 14 % for prehypertrophic cells), in comparison with intact type X (Figure 4A), whereas cell adhesion to both heat-denatured type X and the isolated NC1 domain was decreased to a lesser extent (from 74 to approx. 30 % for hypertrophic and from 41 to approx. 25 % for prehypertrophic respectively)(Figure 4A). Cell binding to heat-denatured collagen indicates that the triple-helical structure is not a strict requirement for cell adhesion, whereas the poor binding to pepsinized type X collagen compared with the isolated NC1 domain may reflect the relative importance of the NC1 domain in cell adhesion. Since chick chondrocytes are capable of binding to the isolated NC1 domain and the interactions appear to be concentration-dependent (Figures 4A and 4C), we examined whether soluble NC1 domain added to the culture medium could competitively inhibit cell adhesion to nX substrate coated on to the plates. It was observed that adhesion of chick cells to type X collagen was dose-dependently inhibited by 10 μg and 20 μg of soluble NC1 domain (Figure 4A), indicating that isolated NC1 domain is capable of competing for cell binding with the intact molecule and that the NC1 domain contains binding site(s) for type X collagen. Adhesion of chondrocytes to denatured NC1 and denatured pepsinized type X collagen were also examined. Figure 4(D) shows that both denatured NC1 and denatured pepsinized type X collagen can support cell adhesion and no significant differences were observed in the percentages of cell adhesion between the native and denatured ligands.

Adhesion of MG63 and HOS cells to pepsinized type X collagen was similarly decreased in comparison with intact type X (Figure 4B). MG63 adhesion to heat-denatured type X and the isolated NC1 domain showed a similar pattern to that observed in chondrocytes, whereas HOS cell adhesion to both heat-denatured type X collagen and the isolated NC1 were similar to that seen for pepsinized type X (Figure 4B). The relatively low level of adhesion of $\alpha 1\beta 1$-integrin-expressing HOS cells to all forms of type X collagen tested and the isolated NC1 domain (as compared with $\alpha 2\beta 1$ expressing MG63 cells) further highlights the importance of the $\alpha 2\beta 1$ integrin in cell–type X collagen interaction.

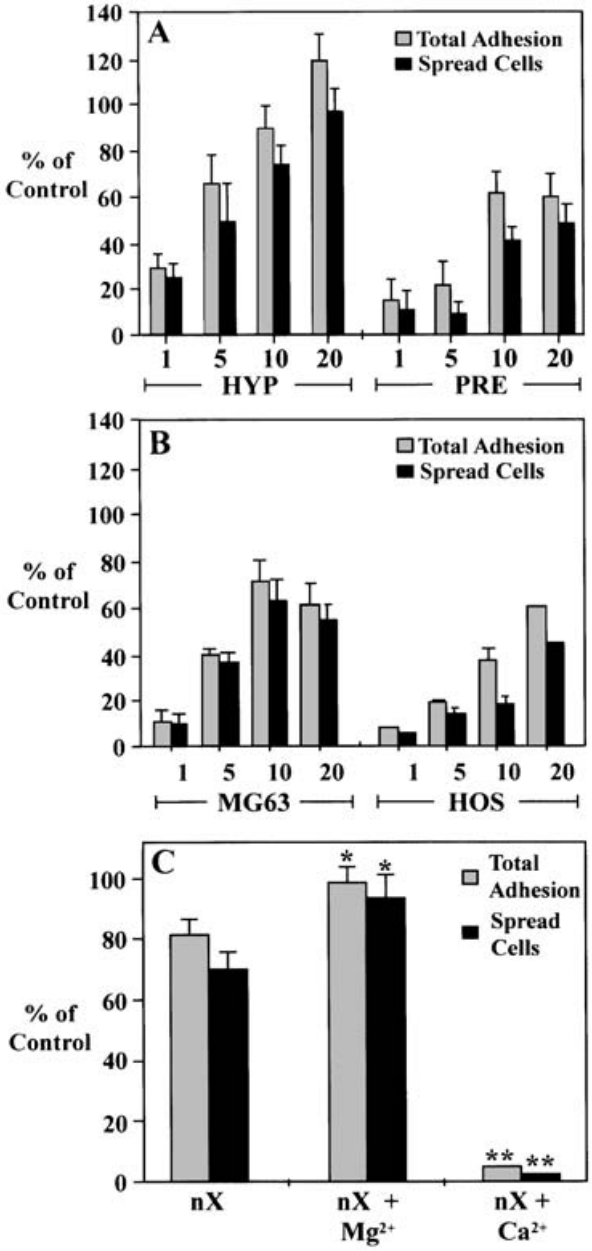

Figure 2 Adhesion of (A) chick hypertrophic (HYP) and prehypertrophic (PRE) chondrocytes and (B) MG63 and HOS cell lines to 1, 5, 10 and 20 μg/well nX, (C) hypertrophic chondrocytes to 10 μg/well nX in the presence of 10 mM Mg^{2+} and 10 mM Ca^{2+}

The diagram shows average readings ± S.E.M. from 3 to 20 adhesion assays of each cell type. Statistical significance: $^{*}P < 0.05$, $^{**}P < 0.005$, as compared with the appropriate adhesion of cells on to type X collagen.

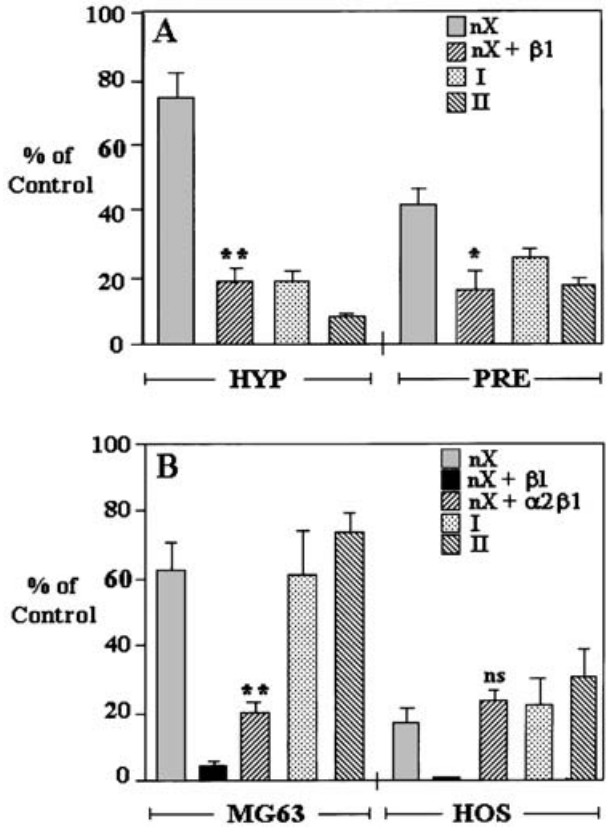

Figure 3 Adhesion profiles of (A) chick hypertrophic (HYP) and prehypertrophic (PRE) chondrocytes to 10 μg/well nX, nX with 1 μg/well function-blocking anti-(chick β1) antibody WB101, types I and II collagens, (B) MG63 and HOS cells to 10 μg/well of nX, nX with 1 μg/well function-blocking anti-(human β1) (Mab13) and anti-$\alpha2\beta1$ antibody (BHA2.1), types I and II collagens

The diagram shows average readings ± S.E.M. from 3 to 20 adhesion assays of each cell type. Statistical significance: $^{*}P < 0.05$, $^{**}P < 0.005$, ns = non-significant, as compared with the appropriate adhesion of cell on type X collagen.

Immunohistochemical studies

Chondrocytes spread on to type X collagen were observed to produce cellular processes and showed the formation of actin stress fibres, as assessed by FITC-conjugated phalloidin staining (Figure 5A). The formation of focal-adhesion plaques was clearly demonstrated by vinculin localization (Figures 5B). No staining was observed in primary (diluent) and serum controls.

Immunolocalizations were also performed with the embryonic epiphyseal cartilage sections of 17-day-old embryonic chick, in which strong immunofluorescence was observed for the β1 and α2 subunits (Figures 6A and 6B). In cultures of hypertrophic chondrocytes, strong immunofluorescent staining was also observed for the β1 and α2 subunits, indicating the presence of these integrin subunits in embryonic chick chondrocytes. The levels of these subunits also appeared to be up-regulated in monolayer culture compared with growth-cartilage tissue sections, as demonstrated by more intense immunofluorescent staining (Figures 6C and 6D).

DISCUSSION

Despite extensive biochemical and genetic studies [15,20,21], the precise function of type X collagen within the extracellular matrix remains undefined. These studies indicate that type X collagen is required for normal development of the growth plate and may have important roles in haematopoietic development [22]. Owing to its unique localization within the epiphyseal growth plate, direct cell–type X collagen interactions may play a pivotal role in maintaining structural integrity of growth-plate ECM and mediate processes in EO. However, such properties have not been investigated. The reported study was initially designed to examine the potential of type X collagen to

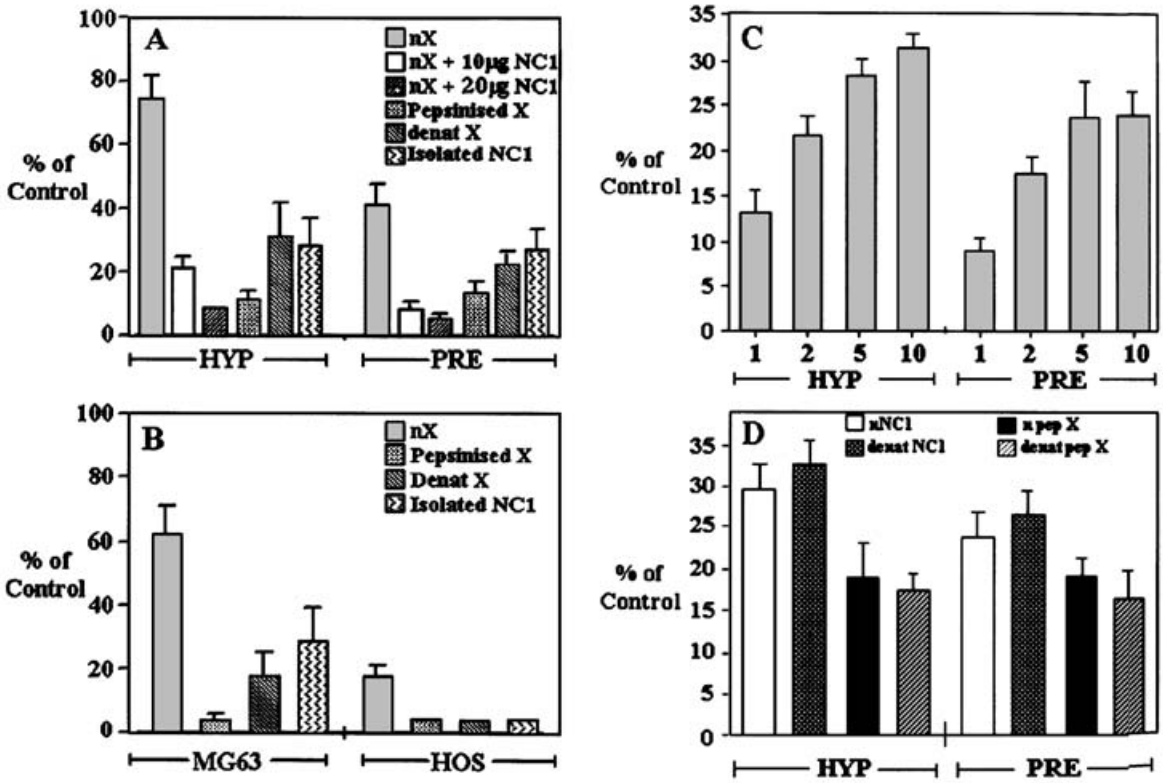

Figure 4 Adhesion profiles of (A) chick hypertrophic (HYP) and prehypertrophic (PRE) chondrocytes to 10 μg/well nX, pepsinized type X (pepsinized X), heat-denatured type X (denat X), 5 μg/well isolated NC1, 10 μg/well nX in the presence of 10 μg and 20 μg of soluble native NC1 domain (NC1), (B) MG63 and HOS cell lines to 10 μg/well nX, pepsinized X, denat X and native NC1, (C) hypertrophic and prehypertrophic chondrocytes to 1, 2, 5 and 10 μg/well native NC1, (D) hypertrophic and prehypertrophic chondrocytes to 10 μg/well native NC1 (nNC1), denatured NC1 (denat NC1), native pepsinized type X collagen (n Pep X) and denatured pepsinized type X collagen (denat pep X)

The diagram shows average readings $\pm$ S.E.M. from 3 to 20 adhesion assays of each cell type.

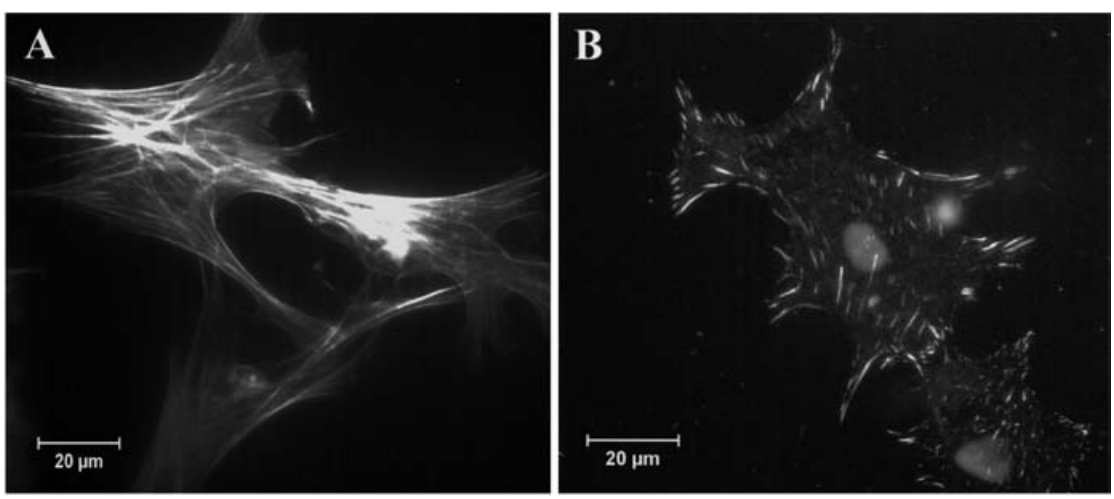

Figure 5 Photomicrographs of chick hypertrophic chondrocytes spread on a type X collagen substrate

(**A**) Visualization of actin stress fibres using FITC-conjugated phalloidin. (**B**) Immunlocalization of vinculin using hVin1 mAb and FITC-conjugated secondary antibody. Cell nuclei were counterstained using propidium iodide. The bar represents 20 μm.

support cell adhesion. The adhesions of both prehypertrophic and hypertrophic chondrocytes to type X collagen were examined in the present study. On the basis of studies with avian tibial dyschondroplasia, it has been suggested that the initial deposition of pericellular type X collagen at the border of transitional and hypertrophic zones is required for the maturation of the

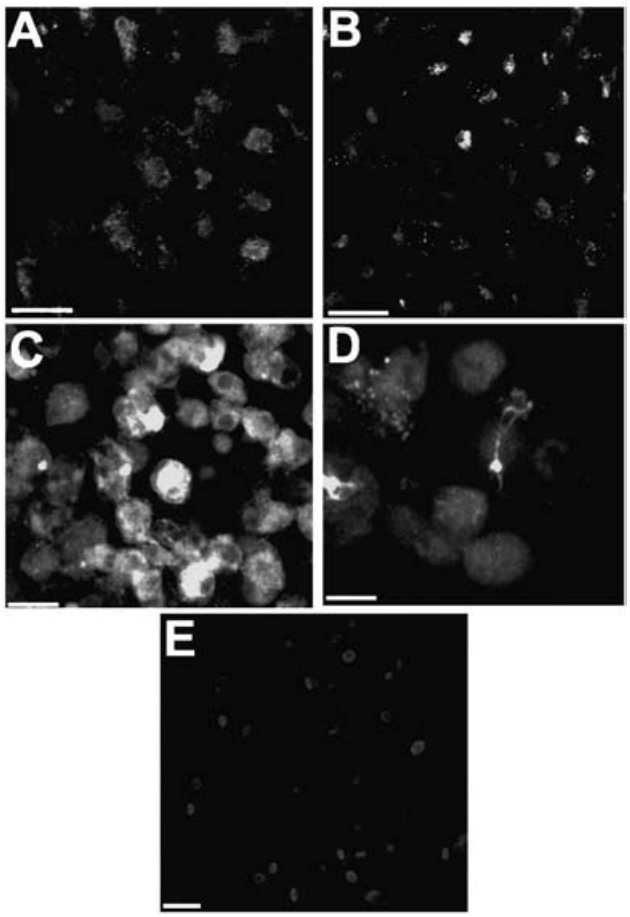

Figure 6 Immunolocalization of integrin subunits in 10 μm cryosections of 17-day-old embryonic chick epiphyseal growth cartilage (hypertrophic region) (A and B) and embryonic tibial hypertrophic chondrocyte cultures (C, D and E)

(**A** and **C**) Localization of the β1 subunit using monoclonal antibody V2E9; (**B** and **D**) localization of the α2 subunit using anti-(chick α2) monoclonal antibody MEP-17; (**E**) control (mouse immunoglobulins replacing primary antibody). Localized α and β subunits were visualized using FITC-conjugated secondary antibodies. Cell nuclei were counterstained using propidium iodide. The bar represents 20 μm.

prehypertrophic chondrocytes [19]. Type X collagen is therefore a relevant substrate for prehypertrophic cells, and the ability of prehypertrophic cell binding to type X collagen was also investigated. The present study has demonstrated that embryonic chick growth-plate chondrocytes are capable of adhering to a type X collagen substrate in a bivalent cation-dependent manner. The enhancement of cell adhesion in the presence of Mg^{2+} indicates the involvement of collagen-binding integrins [8,33], since Mg^{2+} is known to up-regulate $\alpha2\beta1$ integrin activity [34]. This is further supported by the observation that Ca^{2+}, which is known to inhibit integrin-mediated cell adhesion [33], and the anti-$\beta1$ antibody both suppressed chondrocyte adhesion to type X collagen. Furthermore, localization of vinculin in focal contacts [35], and stress-fibre formation, support integrin involvement in cell binding to type X collagen.

Binding of MG63 and HOS was also studied, since they are known to express distinct collagen-binding integrins as detailed above. The observations that MG63 cells, which only express the collagen-binding integrin $\alpha2\beta1$, showed higher levels of adhesion to type X collagen than to HOS cells, which express only $\alpha1\beta1$ integrin, further indicates that type X collagen is a ligand for the $\alpha2\beta1$ integrin. The immunolocalizations of the $\alpha2$ and $\beta1$ integrin subunits in chick epiphyseal cartilage and cultured chondrocytes also suggest possible involvement of $\alpha2\beta1$ in type X collagen–cell interactions. The involvement of other collagen-binding integrins (such as $\alpha1\beta1$ and $\alpha3\beta1$) cannot, however, be excluded. Indeed, both MG63 and HOS cells are known to express $\alpha3\beta1$ integrin [31,32]. This observation may explain the incomplete inhibition of MG63 cell adhesion to type X collagen in the presence of $\alpha2\beta1$-integrin-blocking antibodies, and it is possible that HOS cell adhesion to type X collagen is mediated, at least in part, by $\alpha3\beta1$ integrin. Furthermore, the $\alpha10\beta1$ integrin, which is expressed by chondrocytes, shares significant sequence identity with $\alpha1$ and $\alpha2$ integrin subunits [36] and is a major collagen-binding integrin [37]. The potential involvement of the $\alpha3\beta1$ and $\alpha10\beta1$ integrins in type X collagen binding is therefore worthy of future investigation.

The higher affinity of chick chondrocytes to native type X collagen, as compared with type I and type II collagens, may have biological relevance *in vivo*, in which the pericellular matrix surrounding hypertrophic chondrocytes is particularly rich in type X collagen, and prehypertrophic chondrocytes in the lower transitional zone may also be exposed to type X collagen. In contrast, osteoblastic cell lines (MG63 and HOS), which are unlikely to be exposed to type X collagen, do not display such a high affinity for type X collagen relative to the other collagen types examined. It is apparent from the present study that non-chondrocytic cells possessing the $\alpha2\beta1$ integrin are capable of binding to type X collagen, which is not a relevant substrate for these cell types and therefore it can be argued that such interaction may not have a specific function in the growth plate. However, different cell types are known to express distinct integrin profiles and may therefore respond differently to the same ligand. Further knowledge of other possible integrins involved in type X collagen–cell interactions will no doubt shed light on the specificity of such interaction and its involvement in the processes of EO.

Both heat-denatured and nXs are capable of supporting cell attachment and spreading. These observations suggest that cell–type X collagen interactions are (at least in part) dependent upon the primary sequence. However, the observation that nX supports the greatest level of cell adhesion indicates that the tertiary structure of type X is also important in cell binding. Whilst heat denaturation may have disrupted conformation-dependent cell-adhesion sites, the reported data cannot preclude the possibility that heat denaturation also exposed cell-adhesion sequences that are normally masked in the native molecule. This notion is partially supported by the observations that native and denatured pepsinized type X collagen support chondrocyte adhesions to the same degree. Consequently, the observed interactions between cells and heat-denatured type X collagen may occur via different cell-adhesion receptors. This has already been shown to be the case for several other collagen types [38–41]. Cell-adhesion assays incorporating anti-integrin monoclonal antibodies upon heat-denatured type X collagen-coated plates may provide a clearer explanation for these observations.

Our data also indicate novel cell-adhesion properties of type X collagen, in that the large, globular NC1 domain is involved in mediating the interaction of type X collagen with cells. Indeed, the isolated NC1 domain is capable of supporting cell adhesion (particularly of $\alpha2\beta1$-expressing cells), and blocking chondrocyte adhesion to intact type X collagen when present in soluble form. It should also be noted that pepsinizing type X collagen (removal of the non-collagenous domains) leads to lower levels of cell adhesion for all cell types tested. The NC1 domain therefore appears likely to contain adhesive sequences; however,

whether the NC1 domain is the major site for chondrocyte adhesion remains to be determined. This is relatively unusual, since most collagens, including type II, type IV and type VI, interact directly with $\alpha2\beta1$ via their triple-helical domains [42–44]. However, the interaction between type VII collagen and dermal fibroblasts (via $\alpha2\beta1$ integrin receptors) is mediated by the NC1 domain of type VII [45], and mesengial-cell interaction with type IV collagen (via $\alpha1\beta1$ and $\alpha2\beta1$ integrin receptors) is mediated by both the triple-helical and the NC1 domains [46]. It is conceivable that interactions of cells with the NC1 domain of type X collagen may play a regulatory role in EO. Indeed, the NC1 domains of the basement-membrane collagen types IV, VIII, XV and XVIII have been shown to regulate angiogenesis, cell proliferation and apoptosis [47,48], all of which are important processes in EO. In addition, both native and heat-denatured NC1 supported the same levels of chondrocyte adhesion, further indicating that cell–type X collagen interactions are mediated via the primary sequence as well as the three-dimensional conformation.

The biological roles of type X collagen in the hypertrophic cartilage ECM are still undefined. It has been suggested that the deposition of this collagen provides a permissive matrix for mineralization and vascularization that occurs prior to new bone formation. Different lines of investigations using transgenic mice have led to the proposal of conflicting roles of type X collagen in the growth plate. An earlier study by Rosati et al. [49] using a type-X-collagen-null mutation in transgenic mice has failed to demonstrate any phenotypic changes in the animals, which leads to the suggestion that type X collagen has no apparent function in the growth plate. However, others have recently demonstrated observable phenotypic changes in the type-X-collagen-null mice which include compression of the proliferative and hypertrophic zones of the growth plate, decompartmentalization of the proteoglycans and disruption of the pericellular matrix in the hypertrophic zone [21]. Very similar, but more severe, phenotypes were also reported in transgenic mice with dominant interference mutations for type X collagen [22]. It was also surprising that both murine models showed affected haematopoiesis manifested by altered B- and T-lymphocyte development [50]. These findings indicate that the skeletal–haematopoietic defects in these murine models are the direct and specific consequence of loss of type X collagen function.

In summary, the present study has demonstrated that type X collagen has the potential to interact with growth-plate chondrocytes and that cell binding is mediated through both the helical and the NC1 domains. The binding of chondrocytes in growth-plate cartilage involves the collagen-binding integrin $\alpha2\beta1$. The functional significance of these interactions is not yet known, but it can be postulated that the type X collagen network in the pericellular matrix may act as a link between cells and matrix components which do not have an apparent cell-binding domain. We have recently observed interactions between type X collagen and a number of ECM components including decorin, biglycan and the glycosaminoglycan hyaluronan (A. P. L. Kwan and K. Ferguson, unpublished work). These observations tentatively indicate the stabilization of the proteoglycan network of the hypertrophic cartilage in the growth plate which involves cell–type X collagen interactions. Such interactions between chondrocytes and type X collagen may also elicit cell-signalling events essential to the processes of EO. The present study therefore provides the basis for further study into the role of type X collagen during EO, and has implications for the future study of bone formation in normal and pathological skeletal conditions.

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