at or about stage 5 for the midbrain, and at stage 5 and later stages for somites. We are aware that these suggestions are speculative, but may lead to further meaningful studies.

Methyl groups vs. BrdU action

Experiments conducted in our laboratory have shown that in early chick embryos the BrdU effect could be greatly alleviated by subsequent treatment with excess thymidine or methionine, but not homocysteine (table 2). The precise mode of action of BrdU remains unclear, but the evidence indicates that the specific effects of BrdU stem from non-random and differential substitutions by BrdU in those DNA sequences responsible for the control of specialized cell functions[34–37]. However, BrdU inhibition is not the result of mutagenic action, because it is reversible[8,9,18,20] and no altered proteins can be detected[9]. Since BrdU has a bromine atom substituting for the methyl group of thymidine, and methionine is a major methyl donor for vertebrate cells[38], we have been led to interpret the BrdU effect in terms of the role of methyl groups. Methyl groups are required for the conversion of deoxyuridine 5′-phosphate to thymidine 5′-phosphate in DNA synthesis (figure 4). This conversion requires, in addition to thymidylate synthetase, adequate methylating agents[39]. Methionine, by virtue of its labile methyl group, is known to serve in this capacity[38], thus stimulating the synthesis of thymidine 5′-phosphate (and hence its dephosphorylated form, thymidine). This in turn may be related to the observations that methionine, like thymidine[8–10,18], can reverse the BrdU effect (table 2). The alleviation of BrdU effect by thymidine is correlated with a decreased incorporation of BrdU into DNA[9].

A number of studies have shown that the methyl group of methionine plays an important role in cell differentiation. For example, Parsa et al.[38] reported that 1. acinar cells of the rat pancreatic anlage cultured in vitro requires a certain level of methionine to differentiate; 2. homocysteine, a demethylated derivative of methionine, in equimolar concentrations cannot substitute for methionine to initiate morphologic and enzymatic differentiation in acinar cells. There is also evidence that the distribution of methyl groups in DNA is not random, and transmethylation is indispensable to cell differentiation[40]. Thus the failure of homocysteine to alleviate the BrdU effect appears to be directly attributable to the methyl group. Furthermore, about 30% of exogenous uridine can be incorporated into DNA of chick embryonic cells[19,31]. This incorporation is enhanced as much as 18% by the presence of excess methionine in a developing system[27]. Since BrdU is a competitive inhibitor of thymidine, the observations that uridine cannot alleviate the BrdU action as effectively as thymidine is not unexpected. Tencer and Brachet[13] reported that BrdU-treated amphibian cells were larger in diameter than their untreated counterparts. This finding raises the possibility that the effects of BrdU on cell surface[42] may be mediated by methyl groups (protons)[43].

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Chondrogenesis in chick limb buds and somites*

by H. Holtzer, M. Okayama, J. Biehl and S. Holtzer

Department of Anatomy, School of Medicine, University of Pennsylvania, Philadelphia (Pa. 19174, USA)

The literature dealing with chondrogenesis in embryonic somite cells and limb bud cells is extensive and polarized[1–5]. The central issues are whether the sulfated proteoglycans synthesized and deposited by the definitive chondroblasts are identical to those synthesized by 1. the presumptive chondroblasts, and 2. by cells outside the chondrogenic lineage. Many investigators[6–13] have claimed that presumptive chondroblasts and a variety of nonchondrogenic cells synthesize the same sulfated proteoglycans as are synthesized by definitive chondroblasts. Somite or limb bud cells are claimed to possess an active 'chondrogenic genotype'[14], and the primary differences between presumptive and definitive chondroblasts are visualized as quantitative and incremental rather than qualitative. According to this view, the presumptive chondroblast itself transforms into the definitive chondroblast, the transition requiring merely the enchancement of synthesis of the sulfated proteoglycans which the cell already was producing. This view, that presumptive and definitive chondroblasts are one and the same cell, renders untenable the contention that pre-

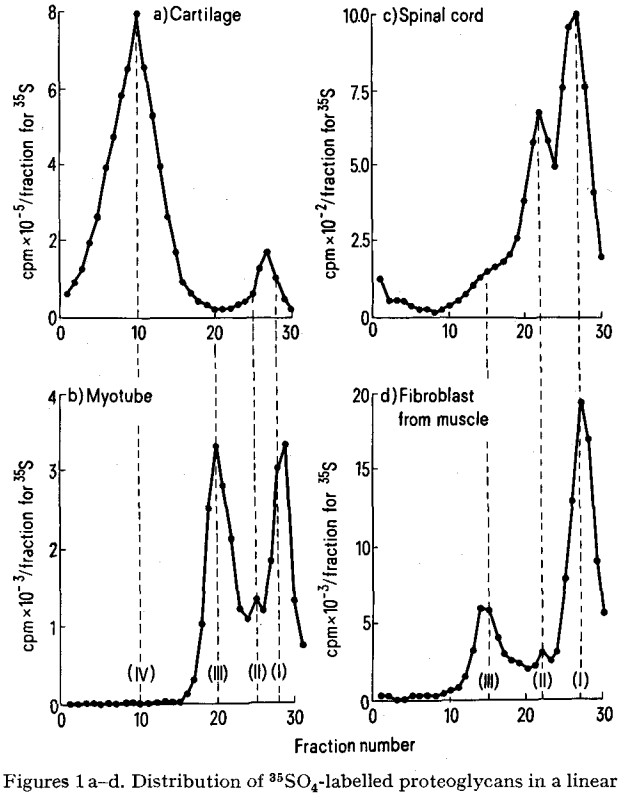
sumptive chondroblasts are obligated to pass through a quantal cell cycle in order to generate daughter, definitive chondroblasts[15,16].

A diametrically opposed view[2,17,18] has long held that, though many cells synthesize sulfated glycosaminoglycans, those sulfated proteoglycans synthesized and deposited as extracellular metachromatic matrix by the definitive chondroblasts are unique, and that such complexes are not synthesized by either presumptive chondroblasts, or by cells outside the chondrogenic lineage. According to this view, the transition between presumptive and definitive chondroblasts involve a qualitative switch in the kinds of sulfated proteoglycans synthesized. Furthermore, it is claimed that such a switch occurs only after a presumptive chondroblast has replicated to yield daughter, definitive chondroblasts: presumptive chondroblasts would not have the option to transform directly into definitive chondroblasts.

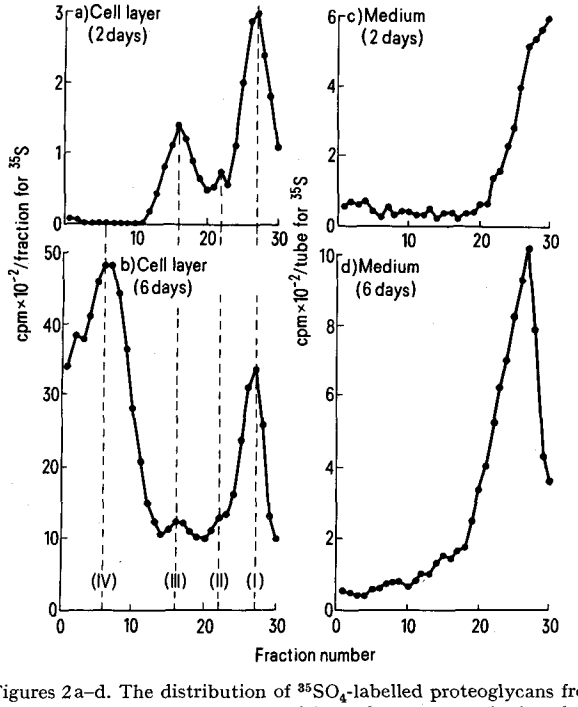
To resolve some of these issues, the sulfated proteoglycans synthesized by presumptive and definitive chondroblasts have been analyzed, using the 4M guanidinium chloride extractions procedure of Hascall and Sajdera[19] and the sucrose gradient procedure of Kimata et al.[20]. The sulfated proteoglycans synthesized by these 2 types of chondrogenic cells have been compared to those synthesized by blastodisc cells, as well as to those synthesized by cells outside the chondrogenic lineage, such as nerve and muscle cells (for more details, Holtzer et al.[21]).

Figures 1a–1d illustrate the differences among those sulfated proteoglycans synthesized by cultured definitive chondroblasts (figure 1a), myotubes (figure 1b), spinal cord cells (figure 1c) and authentic fibroblasts (figure 1d). These different types of cells were grown as described in Abbott et al.[22] and exposed to $^{35}SO_4$= from 30 min to 24 h. In addition, cardiac and smooth muscle cells, as well as BrdU-suppressed chondrogenic cells[16,22], have also been examined. Essentially similar profiles are obtained if the above cell types are incubated in ^{14}C-glucosamine. That the labelled material consists largely of proteochondroitin sulfate is shown by treating peaks I, II, III and IV with chondroitinase ABC. Over 90% of peak IV and approximately 50% of peaks I, II and III are digested by the enzyme.

3-day-old cultures of stage 22 chick limb buds consist largely of presumptive chondroblasts, plus modest



Figures 1a–d. Distribution of $^{35}SO_4$-labelled proteoglycans in a linear sucrose gradient after either 25 h (1a and 1b) or after 30 h (1c and 1d) in centrifuge. The labelled proteoglycans synthesized by 10-day definitive vertebral chondroblasts (figure 1a) synthesize molecules that band only in peaks I and IV. The labelled proteoglycans synthesized by cells in cultures rich in myotubes (figure 1b) band only under peaks I, II and III. The labelled proteoglycans from embryonic spinal cord (figure 1c) or from cultured fibroblasts (figure 1d) were run in the centrifuge for 30 h to define better peak II. Note that only molecules that band under peaks I, II and III are recovered in these 2 types of cultures.

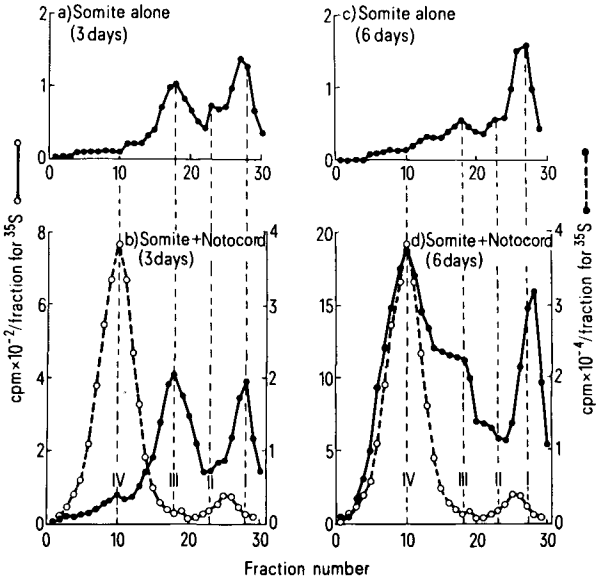


Figures 2a–d. The distribution of $^{35}SO_4$-labelled proteoglycans from limb buds that have been cultured for 2 days and consist largely of mesenchyme cells and presumptive chondroblasts (figure 2a). Compare with the proteoglycans from limb buds that have been cultured for 6 days and consist largely of mesenchyme cells and definitive chondroblasts (figure 2b). The presence of peak IV from limb buds cultured for 6 days correlates with the microscopic appearance of chondroblasts. Figures 2c and 2d, respectively, are the labelled proteoglycans recovered from the medium in which presumptive and definitive chondroblasts were cultured. Approximately 70% of the labelled molecules in the medium are degraded with chondroitinase ABC.

numbers of myogenic cells and presumptive fibroblasts[23]. After 3 days in culture, these cells do not exhibit any metachromatic matrix and EM studies do not reveal cells with the fine structure characteristic of functional, definitive chondroblasts. Figure 2a illustrates that the sulfated proteoglycans synthesized by this population, rich in presumptive chondroblasts, consists of peaks I, II and III. In contrast to 3-day-old cultures, 6-day-old cultures of identical limb buds exhibit large nodules of definitive chondroblasts as determined by light and electron microscopy. The emergence of these definitive chondroblasts is correlated with the appearance of peak IV molecules (figure 2b). The sedimentation profile of peak IV molecules from these older limb bud cultures is indistinguishable from that of peak IV synthesized by the definitive chondroblasts of 10-day chick vertebral cartilages. From these experiments it can be concluded that, contrary to the claims of many investigators[3,6–13], the sulfated proteoglycans synthesized by the presumptive chondroblasts in early limb buds are not the same molecules as those synthesized by the definitive chondroblasts.

Somites from stage 14 chick embryos cultured by themselves do not give rise to definitive chondroblasts; the same aged somites cultured with a piece of spinal cord or notochord for 4–6 days do yield clusters of definitive chondroblasts[2,17,18]. This inductive interaction between spinal cord or notochord and responding somite cells occurs in vivo and in vitro. As shown in figures 3a and 3b, somite cells cultured by themselves for 3 and 6 days synthesize sulfated proteoglycans that band as peaks I, II and III. These cultures, rich in presumptive chondroblasts, do not spontaneously transform into chondroblasts, nor do they spontaneously begin to synthesize peak IV sulfated proteoglycans[2,14,17,25]. Cultures of comparable somites plus a piece of notochord behave differently. By microscopic criteria, 3-day-old cultures of somites plus notochord consist only of presumptive chondroblasts and myogenic cells. Definitive chondroblasts first emerge in these cultures around day 4 or 5. As shown in figures 3b and 3c, the correlation between microscopically detectable metachromatic matrix, and the synthesis of the large molecular weight sulfated proteoglycans of peak IV is striking.

It has been known for many years that a variety of nonchondrogenic cells synthesize sulfated proteoglycans. Figures 1b, 1c and 1d show that the sulfated proteoglycans synthesized by fibroblasts, spinal cord cells and muscle cells are distributed among peaks I, II and III. In other experiments it has been shown that BrdU-



Figures 3a–d. The distribution of $^{35}SO_4$-labelled proteoglycans of somites alone cultured for 3 and 6 days respectively (figures 3a and 3c). These cultures are rich in mesenchyme cells and presumptive chondroblasts; they do not yield any definitive chondroblasts. Cultures of somites plus notochord prior to day 4 do not display molecules that band under peak IV and such cultures are totally wanting in microscopically identifiable chondroblasts (figure 3b). Peak IV molecules are recovered from cultures of somites plus notochord that are older than day 5; such cultures are rich in nascent, definitive chondroblasts (figure 3d).

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suppressed chondrogenic and myogenic cells also synthesize peak I, II and III sulfated proteoglycans.

The experiments described above are incompatible with the repeated claims that presumptive chondroblasts, as well as nonchondrogenic cells, possess an active 'chondrogenic genotype', or that many kinds of embryonic cells turn off their chondrogenic program as they mature or 'stabilize'[3,6–13]. On the contrary, it is more likely that the synthetic program of the definitive chondroblasts is qualitatively distinct from that of its precursor, or mother cell, the presumptive chondroblast.

This stress of difference in synthetic options between presumptive chondroblasts and their daughters, the definitive chondroblasts, is consistent with the finding that only the latter synthesize the cartilage type II collagen chains[26]. Type I collagen is the only type of collagen chain synthesized in early limb bud cells, which consist largely of presumptive chondroblasts. Type II collagen chains are found in limb buds only after the definitive chondroblasts are observed microscopically. From these observations we suggest that a presumptive chondroblast itself does not have the option to transcribe those genes which regulate the synthesis of peak IV sulfated proteoglycans and type II collagen chains. On the other hand, following a quantal cell cycle, a given presumptive chondroblast yields daughter definitive chondroblasts which now have the option to transcribe the genes coding for the core proteins of peak IV sulfated proteoglycans and for type II collagen.

In the absence of an inductive interaction between somites and notochord or spinal cord either in vivo or in vitro, early somite cells fail to chondrify. In a succession of publications Lash and co-workers, and Strudel have variously interpreted this relationship as follows: 1. the notochord secretes a unique molecule that specifically induces undifferentiated somite cells to chondrify[5,27]; 2. somite cells 'spontaneously' transform into chondroblasts[14]; 3. collagen secreted and deposited around the notochord specifically transforms somite cells into chondroblasts[2]; 4. glycosaminoglycans synthesized and released by the notochord is the specific inducing molecule for chondrogenesis[29]; 5. committed chondrogenic cells migrate to the surface of the notochord and there chondrify[25]; and 6. local K ion concentrations are critical for such inductive activity[30].

In contrast to any of these explanations, Holtzer and co-workers[2,17,18,21,23] have suggested that the above experiments merely demonstrate that enrichment of the culture medium in which somites are growing will permit them to chondrify, providing that their past mitotic history has rendered them competent to do so. More specifically, the notochord or spinal cord permits endogenously programmed presumptive chondroblasts to undergo the quantal cell cycle that yields daughter definitive chondroblasts. The definitive chondroblasts are the only cells in, or outside of, the chondrogenic lineage with the option to transcribe and translate all of the proteins required for those sulfated proteoglycans that band under peak IV and that synthesize type II collagens chains. Simple exposure of presumptive chondroblasts or 'somite' cells or 'limb bud cells' to culture media enriched with exogenous glycosaminoglycans or collagen chains or to analogs of nicotinamide[13] cannot induce such cells to switch to the synthetic program characteristic of definitive chondroblasts. The most such exogenous molecules can do is to permit presumptive chondroblasts which divide in their presence to undergo the quantal cell cycle which yields definitive chondroblasts. As such, exogenous molecules play the relatively trivial role of 'trigger' rather than delivering information which instructs cells how to diversify.

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Yolk sac erythropoiesis

by Françoise Dieterlen-Lièvre

Institut d'Embryologie du C. N. R. S. et du Collège de France, 49bis, avenue de la Belle Gabrielle, F-94130 Nogent-sur-Marne (France)

The yolk-sac is an extraembryonic appendage with 2 functions: absorption of the yolk nutrients and genesis of blood cells during embryonic life. The blood cells produced are mainly erythrocytes. The erythropoietic function is especially striking in birds, because of its duration and the large number of cells formed. However, these 2 parameters can vary considerably among species, depending on the relative roles in haemopoiesis of other organs such as liver, spleen and bone marrow. In the chick, which has been the most studied species, the liver has no erythropoietic function and the yolk sac (YS) is active for the greater part of embryonic life[1].