Investigations into mechanisms of amino acid supply to the rat embryo using whole-embryo culture

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ABSTRACT The technique pioneered by D.A.T. New for the *in vitro* culture of early post-implantation rat embryos has been used to study nutritional mechanisms during early organogenesis. The results indicate that the principal route for amino acid supply to the 8.5- to 11.5-day embryo involves the endocytosis of proteins into cells of the visceral yolk sac endoderm, their digestion in lysosomes, and transmission of the amino acids to the growing embryo. Free amino acids constitute a comparatively unimportant source. Inhibition of either endocytosis or intralysosomal proteolysis diminishes amino acid supply to the embryo, and this can result in embryonic death or maldevelopment during organogenesis.

KEY WORDS: *visceral yolk sac, amino acid, embryonic nutrition*

Of the many models used to study embryonic development *in vitro*, the technique of whole-embryo culture developed by Denis New (reviewed by New, 1978) has been particularly useful. The primary advantage of New's technique is that normal growth and development is achieved during that period of organogenesis after implantation but before the chorioallantoic placenta is established, presumably because the serum commonly used as medium mimics closely the environment to which the conceptus is exposed *in utero*. The technique provides investigators with a unique opportunity to study the mechanisms whereby specific factors influence *in vitro* development of the embryo and, as discussed below, to evaluate the importance of these processes *in utero*.

A major theme of our work over the past 15 years has been an exploration of the role of the rat visceral yolk sac in supporting normal embryonic development by the processing of nutrients. Jollie (1990) and Lloyd (1990) reviewed early work on the structure and function of the rodent yolk sac placenta; Beckman *et al.* (1990a) and Brent *et al.* (1990) discussed how yolk sac dysfunction can be a cause of abnormal embryonic development. The whole-embryo culture technique developed by New and his colleagues has been central to several recent investigations which have shown that the visceral yolk sac is the primary supplier of amino acids for the embryo and that amino acids are generated in the visceral yolk sac lysosomes by digestion of endocytosed protein.

Using whole-embryo culture initiated on 9.5 days post-conception, Cockroft (1979) obtained morphologically normal rat embryos after culture in 100% rat serum. He then dialyzed rat serum to remove its low molecular weight components and showed that conceptuses cultured in the dialyzed rat serum grew and developed well only if it was supplemented with a mixture of vitamins and glucose. The absence of free amino acids from the dialyzed serum seemed of little consequence. Although it had been postulated (Beck and Lloyd, 1966) that yolk sac-mediated digestion of macromolecules was necessary for the support of normal embryonic development during organogenesis, Cockroft's data supported this hypothesis in respect of a specific nutritional factor, the supply of amino acids.

Whole-embryo culture experiments (Freeman *et al.*, 1981; Freeman and Lloyd 1983a) confirmed the hypothesis that the visceral yolk sac supplies amino acids to the embryo by a process involving pinocytosis and lysosomal digestion of serum proteins. In these studies, cultures were initiated on 9.5-days post-conception in rat serum containing ^{3}H-labeled rat serum proteins. After 24 h, analysis of tissues of the conceptus showed that the visceral yolk sac had digested these proteins and the resulting amino acids had been utilized for the synthesis of embryonic tissue protein. Subsequently, Rowe and Kalaizis (1985), investigating serine metabolism in the cultured 9.5-day rat embryo, indirectly estimated that the majority, about 86%, of serine utilized by the embryo was supplied by this process.

Later, we reported a direct determination of the relative contribution of yolk sac-mediated protein digestion to the supply of amino acid to the embryo (Beckman *et al.*, 1990b). As part of this investigation, 9.5-day rat conceptuses were cultured in the presence of both free [^{3}H]leucine or rat serum proteins containing [^{3}H]leucine. The accumulation of radioactivity into

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TABLE 1

SPECIFIC RADIOACTIVITY OF [^{3}H]LEUCINE IN THE ACID-INSOLUBLE FRACTION OF RAT EMBRYOS FOLLOWING CULTURE IN THE PRESENCE OF FREE [^{3}H]LEUCINE OR PROTEINS CONTAINING [^{3}H]LEUCINE

Specific radioactivity in culture medium	Specific radioactivity in acid-insoluble fraction of embryo	Ratio of specific radioactivities (acid-insoluble fraction/ culture medium)
Source: free [^{3}H]leucine		
2470	36	0.014
2810	25	0.009
2470	22	0.009
2790	14	0.005
2950	15	0.005
		Mean= 0.008
Source: proteins containing [^{3}H]leucine		
2.21	0.92	0.416
2.67	1.17	0.438
1.16	0.80	0.690
1.28	1.34	1.047
1.64	0.76	0.463
		Mean= 0.611

Whole embryo culture was initiated on 9.5 days post-conception and continued for 24 h in culture medium containing free [^{3}H]leucine or rat serum proteins containing [^{3}H]leucine. The specific radioactivities of [^{3}H]leucine in the culture media and the acid-insoluble fraction of embryos are expressed as pCi of ^{3}H per nmole of leucine. The results of 5 separate experiments are shown. Data from Beckman *et al.* (1991b).

tissue proteins by the visceral yolk sac and the embryo was determined and expressed as a clearance. [Clearance was defined as the μl of culture medium cleared of radioactivity per milligram of tissue protein (Williams *et al.*, 1975)]. After taking into account the abundance in rat serum of leucine as free amino acid and as protein, it was calculated that over 95% of the leucine utilized for *de novo* synthesis of embryonic tissue protein was supplied by the digestion of protein taken up by the visceral yolk sac.

In a second series of similar experiments, we measured leucine specific radioactivity in the culture medium and in embryonic protein (Beckman *et al.*, 1991b). After equilibrium conditions were attained in the whole-embryo culture, such that a constant value for the specific radioactivity of leucine was reached in the free amino acid pool in the embryo, a less than two-fold dilution of specific radioactivity in the acid-insoluble fraction of the embryo, as compared with that in the serum, was observed when the source present in the culture medium was serum proteins containing [^{3}H]leucine (Table 1). When the source was free [^{3}H]leucine, the dilution of specific radioactivity was 125-fold. This is the expected result if most of the amino acids utilized for the synthesis of embryonic proteins are supplied by yolk sac-mediated protein digestion.

Because we were employing whole-embryo culture, we were also able to study the exchange of amino acids with culture medium (Beckman *et al.*, 1991b). Whole-embryo culture was initiated at 9.5 days post-conception in the presence of free [^{3}H]leucine. After 24 h, conceptuses were washed and culture was continued in fresh serum lacking radiolabeled leucine. Conceptuses were harvested at different times during the next 24 h and analyzed for radioactivity. Surprisingly, the radioactivity per embryo did not decrease substantially during the 24-hour period of culture in the absence of [^{3}H]leucine. This result suggested that the intensely anabolic embryo conserves its amino acids and does not release them back into external amino acid pools.

Subsequently, we were able to utilize whole-embryo culture to ask whether other amino acids were supplied in a manner similar to that determined for leucine. This question originated in part from our concern that leucine may not be a prototypical amino acid in respect to the mechanisms by which the embryo is supplied with amino acids; and in part by reports that reduced availability of free methionine in heterologous serum used for culturing rat conceptuses may result in abnormal development (Flynn *et al.*, 1987; Coelho *et al.*, 1989; Coelho and Klein, 1990).

To answer this question, whole-embryo cultures were initiated on 9.5 days post-conception. Culture serum was supplemented with either free [^{3}H]leucine, serum proteins containing [^{3}H]leucine, free [^{3}H]methionine or serum proteins containing [^{3}H]methionine. Conceptuses were harvested after 24 h of culture and the radioactivity present in the acid-soluble and acid-insoluble fractions of the embryo and visceral yolk sac was expressed as a clearance. When conceptuses were cultured in serum with either free [^{3}H]leucine or free [^{3}H]methionine present, leucine was used almost exclusively for synthesizing protein,

TABLE 2

ESTIMATED CONTRIBUTION OF FREE AMINO ACID AND PROTEIN TO THE SUPPLY OF LEUCINE AND METHIONINE UTILIZED FOR PROTEIN SYNTHESIS BY THE CULTURED RAT EMBRYO

Amino acid	source	24 h clearance into acid-insoluble fraction (μl per mg protein)	concentration in serum nmoles per μl)	24 h incorporation into protein (nmoles per mg protein)	percentage of total incorporation
leucine	free amino acid	252	0.122	30.7	3.2
	serum proteins	24	38.18	916.3	96.8
methionine	free amino acid	139	0.04	5.6	5.5
	serum proteins	27	3.56	96.1	94.5

Whole embryo culture was initiated on 9.5 days post-conception and continued for 24 h in culture medium containing free [^{3}H]leucine, free [^{3}H]methionine, serum proteins containing [^{3}H]leucine or serum proteins containing [^{3}H]methionine. Data from Lloyd *et al.* (1996).

whereas a small but significant fraction of the methionine was found in the acid-soluble (low molecular weight) fraction (Lloyd *et al.*, 1996).

When conceptuses were cultured in the presence of serum proteins containing radiolabeled amino acids, serum proteins were taken up and digested supplying both leucine and methionine to support *de novo* synthesis of tissue protein. Again, a significantly greater fraction of the methionine incorporated was found in the low molecular weight fraction.

To estimate the contributions of free amino acid and protein to the supply of leucine and methionine utilized for protein synthesis by cultured 9.5- to 10.5-day rat embryos, values of clearance in the acid-insoluble fraction of the embryo were compared with published values of amino acid concentrations in serum, either in free amino acid or in protein, to calculate the net 24-hour incorporation into embryonic protein. The net incorporation from free amino acids and protein was then expressed as a percentage of the total incorporation (Table 2). These calculations show that, as with leucine, the majority of methionine taken up by the embryo and utilized for protein synthesis is supplied by protein digested in the visceral yolk sac.

New's whole-embryo culture technique most commonly uses rat embryos explanted on 9.5 days post-conception and harvested approximately 24 or 48 h later, at a stage when the visceral yolk sac surrounds the embryo and the two structures are interconnected by the vitelline circulation. We hypothesized that, in the 8.5-day embryo, protein digested by the visceral yolk sac may not be as critical for the supply of amino acids, because the visceral yolk sac does not surround the embryo at this stage of development. Instead, the embryonic endoderm is in contact with the culture medium, and the vitelline circulation has not yet formed. We tested our hypothesis using the method described by New and colleagues (Buckley *et al.*, 1978) for the culture of 8.5-day rat embryos. After 24 h in the presence of either radiolabeled amino acids or serum proteins containing radiolabeled amino acids, the patterns of incorporation of radioactivity into tissues of the conceptus led us to conclude that, despite the different anatomic relationships, the majority of leucine and methionine incorporated into proteins of the conceptus was supplied by the digestion of protein, most likely in the visceral yolk sac (Beckman *et al.*, 1996).

The technique of whole-embryo culture has enabled us to characterize a mechanism by which the 8.5- to 11.5-day rat embryo is supplied with amino acids. The relative contribution of this process to the total supply of amino acids *in vitro* has been demonstrated directly and the importance of this process to normal embryonic development *in vivo* has been examined by us and others. Additionally, various inhibitors of yolk sac function have been shown to result in abnormal development, reduced growth or embryonic death in whole-embryo culture, including anti-visceral yolk sac serum (Freeman *et al.*, 1982; Beckman *et al.*, 1990b, 1991a), suramin (Freeman and Lloyd, 1986), and leupeptin (Freeman and Lloyd, 1983b). When examined *in vivo*, the effects of these agents on development mirrored those seen *in vitro* (Freeman and Lloyd, 1983b, 1986; Brent *et al.*, 1990; Beckman *et al.*, 1991a). Thus whole-embryo culture has been utilized to characterize and quantify yolk sac-mediated protein digestion, a process that appears equally important to the embryo developing *in vivo*.

This brief summary of a facet of our laboratory's work demonstrates one application of Denis New's whole-embryo culture technique. We believe the technique has provided and will continue to provide opportunities for understanding developmental processes.

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