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Prolonged in vitro Maintenance of Human Diploid Fibroblasts in a New Tissue Culture Medium (PRC-1)

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Abstract. A new medium, designated PRC-1, has been developed that (a) will support proliferation of cells outgrown from human tissue explants and (b) will maintain in culture for prolonged periods human diploid fibroblasts derived from normal, benign, or tumor stromal tissue specimens. Of the 51 tissue specimens used, 28 fibroblastic lines were produced which exceeded their expected life span and lived in the PRC-1 medium for longer than 7 months and up to 25 months (9–91 1:4 subcultivations), as contrasted with lines from the same specimens which were started and maintained in other media (McCoy's 5a, Ham's F12, MEM and Leibovitz L15). All lines of fibroblastic morphology retained their diploid chromosomal mode throughout their entire serial maintenance in vitro.

Introduction

The vast variety of media used in tissue culture implies that both quantitative and qualitative compositional differences play significant roles in their capacities of growth facilitation and the in vitro maintenance for specific cell types. Since several media possess similar qualitative but differing quantitative compositions [1], it could be inferred that concentrational alterations of certain components might provide nutritional combinations which could fulfill the requirements of specific cell types for their prolonged in vitro maintenance [2–5]. Additionally, it should be mentioned that most physiological compounds can become inhibitory to in vitro cell growth when used at high concentrations due to the possibility of physical and biochemical interference with cellular metabolism. Conversely though, a reduced supply or the total absence of certain compounds or ions might decrease or inhibit the ability of cells to grow by urging the cells to use other metabolic pathways that eventually lead to the cessation of growth. Determining the optimal balances among medium components, therefore, becomes especially difficult when dealing with a complicated multimixture of biologically active compounds [1]. Nevertheless, the development of new media has been progressing, assisted by (a) the in-

Table I. Composition of medium PRC-1

Component	mg/l	mol/l
Amino acids		
L-Alanine	19.12	2.1459×10^{-4}
L-Arginine HCl	115.24	5.469×10^{-4}
L-Asparagine H_2O	24.004	1.5992×10^{-4}
L-Aspartic acid	13.31	1.0×10^{-4}
L-Cysteine	24.00	1.3667×10^{-4}
L-Cystine	4.00	0.1664×10^{-4}
L-Glutamic acid	44.72	3.0401×10^{-4}
L-Glutamine	166.10	1.1361×10^{-3}
Glycine	16.00	2.1304×10^{-4}
L-Histidine HCl·H_2O	20.768	0.9908×10^{-4}
L-Hydroxyproline	9.88	0.7536×10^{-4}
L-Isoleucine	25.32	1.9298×10^{-4}
L-Leucine	44.984	3.4286×10^{-4}
L-Lysine HCl	43.20	2.3645×10^{-4}
L-Methionine	13.752	0.9217×10^{-4}
L-Phenylalanine	18.584	1.1249×10^{-4}
L-Proline	28.72	2.4952×10^{-4}
L-Serine	24.72	2.3520×10^{-4}
L-Threonine	23.92	2.0083×10^{-4}
L-Tryptophan	6.056	0.2965×10^{-4}
L-Tyrosine	17.40	0.9602×10^{-4}
L-Valine	21.72	1.8548×10^{-4}
Vitamins		
p-Aminobenzoic acid	0.41	2.9905×10^{-6}
Ascorbic acid	0.21	1.1925×10^{-6}
d-Biotin	0.0849	0.3474×10^{-6}
Calciferol (D_2)	0.02	0.0504×10^{-6}
Dihydrofolic acid	4.522	1.0198×10^{-5}
DL-α-lipoic acid	0.084	0.4071×10^{-6}
Menaphthone (K_3)	0.002	1.1614×10^{-8}
Nicotinamide	0.2198	1.8001×10^{-6}
Nicotinic acid	0.205	1.6653×10^{-6}
D-Pantothenic acid hemi-calcium salt	0.274	1.0620×10^{-6}
Pyridoxal HCl	0.205	1.0068×10^{-6}
Pyridoxine HCl	0.453	2.2022×10^{-6}
Riboflavin	0.0972	0.2582×10^{-6}
Thiamine HCl	0.218	2.9647×10^{-6}
Vitamin A_1 acetate	0.02	0.4667×10^{-7}
Vitamin B_{12}	0.672	0.4952×10^{-6}

Component	mg/l	mol/l
Other organic components		
Adenine HCl	1.02	0.5944×10^{-5}
Adenosine 5′-monophosphate sodium salt	0.04	1.0805×10^{-7}
Adenosine 5′-triphosphate disodium salt	1.00	2.7013×10^{-6}
Bactopeptone	240.00	
Cholesterol	0.04	1.0346×10^{-7}
Choline chloride	7.684	5.6128×10^{-5}
2-Deoxy-*D*-ribose	0.10	7.4571×10^{-7}
Glucose	2.120.8	1.1782×10^{-2}
Glutathione reduced form	0.21	6.8337×10^{-7}
Guanine HCl	0.06	3.1982×10^{-7}
Hypoxanthine sodium salt	1.968	1.2369×10^{-5}
i-Inositol	21.61	1.1992×10^{-4}
Linoleic acid	0.0336	0.1198×10^{-6}
Phenol red	5.0	1.3285×10^{-5}
Putrescine 2 HCl	0.0644	1.1992×10^{-6}
D-Ribose	0.1	0.6662×10^{-6}
Sodium pyruvate	44.0	3.9963×10^{-4}
Thymidine	0.292	1.2056×10^{-6}
Thymine	0.06	4.7581×10^{-7}
Tween 80	1.0	
Uracil	0.06	5.3523×10^{-7}
Xanthine	0.06	3.9447×10^{-7}
Inorganic salts		
$CaCl_2$	93.26	8.4720×10^{-4}
$CuSO_4 \cdot 5H_2O$	0.0005	0.2002×10^{-8}
$Fe(NO_3)_3 \cdot 9H_2O$	0.144	3.5656×10^{-7}
KCl	330.0	4.4265×10^{-3}
$MgSO_4 \cdot 7H_2O$	120.0	4.8706×10^{-4}
NaCl	6.983.6	1.1948×10^{-1}
$NaHCO_3$	1.570.0	1.8627×10^{-2}
$Na_2HPO_4 \cdot 7H_2O$	53.6	0.2×10^{-3}
$NaH_2PO_4 \cdot H_2O$	258.5	1.8736×10^{-3}
$ZnSO_4 \cdot 7H_2O$	0.1726	0.5993×10^{-6}
Antibiotics		
Penicillin	10^5 IU/l	
Streptomycin	100 mg/l	
Amphotericin B	2 mg/l	

creasing knowledge of specific qualitative and quantitative cell requirements, (b) the advances of experience gained through the routine utilization of existing media, and (c) subjective and empirical judgement. In the present report, a new tissue culture medium is described which facilitated the in vitro maintenance of diploid fibroblasts derived from human tissue specimens for significantly longer periods, as compared with other media.

Materials and Methods

Medium

Table I shows the composition of the PRC-1 medium. The medium was prepared following the detailed instructions of *Ham* [6] and of *Shipley and Ham* [7]. Fetal bovine serum (Flow, Irving, Scotland), as supplied without heat inactivation, was used at a concentration of 15–20% at the beginning of a culture, and after the first month at 10%. For the preparation of medium and trypsin solutions, tap water was first passed through a 1.5-m Zalion deionizing column (Ionel, Athens, Greece) and then double-distilled using a whole-glass Fontavapor 285 distillation device (Büchi, Flawil, Switzerland). All component chemicals were of the purest grade available. Chemicals were purchased from Sigma (St. Louis, Mo.), Mallinckrodt (St. Louis, Mo.), or Merck (Darmstadt, FRG).

Trypsin Solution

Crystalline trypsin (Difco, Detroit, Mich.) was dissolved in a solution containing KCl 0.09 *M*, NaCl 0.009 *M*, *D*-glucose 0.045 *M*, tris-(hydroxymethyl)-aminomethane 0.05 *M*, and phenol red 0.02 g/l [8]. pH was adjusted to 7.7–7.8 at 37 °C and the solution filtered through a 0.22-m sterilizing filter (Millipore, Bedford, Mass.) and stored at 5 °C. The lowest effective concentration used for human fibroblasts was 1 g/l.

Cell Cultures

Human fibroblast cultures were derived from tissue specimens as previously described [9]. Cells were routinely maintained in both PRC-1 and McCoy's 5a [10] media, as well as in other media occasionally, Ham's F12 [6], MEM [11], Leibovitz L15 [12], for purposes of comparison. Confluent cultures were subcultivated at a ratio of 1:2 for the first and second subcultivations, and thereafter at 1:4, routinely. Plastic flasks (Nunk, Denmark) were used. Flasks were gassed with a sterile mixture of 5% CO_2 in air. The medium was changed twice a week. An incubator (New Brunswick Scientific, N.J.), adjusted to 36.6 °C, was used. All cell cultures described were free of mycoplasma and other contaminants as examined by ^{3}H-thymidine labelling, autoradiography, and microscopy [13]. Metaphases were prepared as previously described [9], routinely every 1–3 months in all cultures. Known commercial media were purchased from Flow (Irving, Scotland).

Results and Discussion

The new PRC-1 medium was developed as a consequence of our initial purpose to investigate the possibility of increasing the efficiency of propagation in culture of cells from specimens of breast tissue [9]. The composition of the PRC-1 medium was determined in order to provide a full range of all essential biological molecules at concentration ranges known to be noninhibitory and yet supportive of growth of several different cell types.

Particular features of the new medium (table I) include: (a) presence of purine and pyrimidine bases, certain nucleotides and ribose and deoxyribose at 5 times lower concentrations than those used in medium 199 [14]; (b) all inorganic elements contained in Ham's F12 [6] were included at 5 times lower concentration, except for $CaCl_2$ which was increased. $FeSO_4 \cdot 7H_2O$ was substituted by $Fe(NO_3)_3 \cdot 9H_2O$; (c) a complete series of essential and nonessential amino acids were used at concentrations higher than those used in Ham's F12 [6], but lower than the DMEM [15], approximating nearer the concentra-

tions used in McCoy's 5a [10]: (d) bactopeptone was also included since it promotes the propagation of primary cell lines [10, 16]: (e) the osmolarity of the PRC-1 medium is 288 mOsm/kg.

For the purposes of this study, it would be a practical impossibility to test for extended durations and in such a vast variety of specimens, the several variants of new media, differing in the concentration and combination

Table II. Efficiency of the medium PRC-1 in maintaining in vitro growth of human fibroblastic cells as compared with other media[1]

Cell culture designation	Case and site of specimen derivation	Months in serial cultivation and (total number of 1:4 subcultivations) in				
		PRC-1	McCoy's 5a	Ham's F12	MEM	Leibovitz L15
AK	trachelitis	19 (31)	6 (7)	ND	ND	ND
AL-BC	CaM – primary tumor	8 (27)	7 (19)	7 (25)	ND	ND
AL-T-BC	CaM – primary tumor	17 (49)	6 (18)	ND	ND	0 (0)
AND-BC	CaM – primary tumor	7 (14)	0 (0)	4 (11)	ND	ND
AND-TH	CaM – nipple	20 (42)	2 (3)	ND	ND	ND
CHA-EP	CaM – normal breast epidermis	18 (67)	7 (27)	ND	6 (25)	5 (21)
CHA-L	CaM – metastatic lymph node	7 (26)	7 (24)	ND	7 (24)	5 (21)
CI	endometrial leiomyoma	14 (42)	5 (16)	ND	ND	ND
DA-ME	NM – primary tumor	22 (42)	5 (12)	ND	ND	6 (11)
DA-T-ME	NM – primary tumor	19 (40)	7 (18)	ND	ND	6 (12)
EL-M	M – primary lesion	9 (27)	0 (0)	0 (0)	ND	ND
G-BC	CaM – primary tumor	9 (17)	0 (0)	0 (0)	ND	ND
G-EP	CaM – normal breast epidermis	23 (84)	6 (28)	9 (36)	ND	ND
G-L	CaM – metastatic lymph node	14 (52)	7 (30)	7 (31)	ND	ND
GA-NB	normal breast epidermis	10 (32)	0 (0)	7 (22)	ND	ND
KA-BC	CaM – primary tumor	9 (20)	ND	4 (11)	ND	ND
KAL-BC	Ca – primary tumor	4 (12)	2 (2)	ND	ND	ND
KR-BC	Ca – primary tumor	10 (17)	2 (2)	ND	ND	ND
LE-M	M – primary lesion	10 (26)	9 (24)	0 (0)	ND	ND
MI-M	M – primary lesion	12 (42)	10 (25)	ND	ND	ND
PAP-M	M – primary lesion	18 (52)	0 (0)	0 (0)	2 (0)	ND
PG-M	M – primary lesion	22 (88)	2 (2)	2 (0)	0 (0)	ND
PL-BC	CaM – primary tumor	8 (23)	4 (12)	4 (11)	ND	ND
P-NPC	nasopharyngeal carcinoma	13 (41)	0 (0)	8 (19)	ND	ND
SE-L	CaM – metastatic lymph node	25 (91)	2 (2)	7 (34)	ND	ND
ST-BC	CaM – primary tumor	9 (34)	7 (22)	6 (21)	ND	ND
SX-F	CaM – primary tumor	10 (9)	2 (2)	4 (9)	ND	ND
TA-N	tonsillitis	17 (54)	5 (18)	ND	ND	ND
TONS-F	tonsillitis	14 (24)	8 (14)	ND	6 (12)	ND

CaM = infiltrating ductal carcinoma with metastases in lymph nodes; Ca = infiltrating ductal carcinoma without metastases; M = chronic mastitis; NM = nipple melanoma; PRC-1 = Papanikolaou Res. Ctr. medium 1; MEM = minimum essential medium of Eagle; ND = not done.

[1] Finite lines maintained for shorter than 3 months are not included.

of their ingredients, e.g. according to such procedures as those reported for improving clonal growth of cells [4, 5]. It was therefore decided to plan and prepare the new medium, and then to compare its efficiency with other known media. Thus, equal proportions of tissue samples, identically processed for tissue culture as previously described [9], were transferred in different media. All flasks were examined weekly for the presence of cell outgrowth from the tissue pieces, or for the formation of small colonies from isolated cells. Specimens which demonstrated no signs of growth for 40–50 days were considered unsuccessful and were discarded.

Table II shows the results of comparisons made with several media and the PRC-1 medium to determine the efficiency of each to maintain for extended durations the growth of fibroblastic cells derived from breast tissues and a limited number of other specimens. During the course of this work the following observations were made:

(1) Most specimens were giving growth of slowly proliferating epithelial cell colonies after 6–15 days in culture. This was followed by an active overgrowth of fibroblasts so that epithelial cells were serially diluted during passaging and eventually disappeared. Attempts made to isolate the colonies of epithelial cells by trypsinization and local sucking with a fine-end Pasteur pipette were usually unsuccessful. In 6 out of 19 cases, this procedure gave epithelial-like finite lines [17, and unpublished results] which were free of fibroblastic cells. All other finite lines were of fibroblastic morphology, and their growth and other properties have been previously described [9, 18, 19]. No morphological changes were noted in the cells throughout their serial cultivation in the PRC-1 medium.

(2) Change from PRC-1 to other media did not influence cell morphology or growth properties as observed for periods of up to 15 days, though certain media would not facilitate prolonged maintenance if used from the start.

(3) 28 fibroblastic lines produced out of 51 tissue specimens were maintained in vitro for periods longer than 7 months and up to 25 months, subjected to 9–91 1:4 subcultivations (table II). The remaining 23 tissue specimens failed to give cell growth in all media. Other media used showed either little or no ability to facilitate growth for periods longer than 7 months. The limit of approximately 7 months is representative of the normal life span of human diploid fibroblasts in vitro, as cultured in MEM [20–26]. A finding of the present work was the prolonged maintenance of five lines: two lines from normal breast epidermis of cancer patients (CHA-EP, G-EP), one line produced from breast epidermis of a healthy donor (GA-NB), and two lines produced from excised tonsils (TA-N, TONS-F). As can be seen from table II, lines derived from different patients with the same, or a different, disease can be maintained for variable time periods in vitro. The finite longevity and the capacity for division may, therefore, also depend on the fulfillment of specific nutritional requirements of the corresponding specimen. Thus, the medium PRC-1 was shown to increase the life longevity of both normal and tumor-derived stromal fibroblasts in vitro. This may be an acquired property as a result of the in vitro establishment, or a differential programming of the finite capacity of these cells to divide. This phenomenon should be discriminated from the spontaneously induced proliferation in culture [9, 27, 28]. On the other hand, human skin fibroblasts can survive in vitro

for longer or shorter times than expected and also can show growth abnormalities, depending on the depth of the tissue layer from which they originate [29, 30] and on the pathology of the donor [31–33].

(4) The chromosomal number in all fibroblastic lines was within the ranges of the normal diploid mode [20–22]. In some cases, double minutes were observed at varied low frequencies [19]. Also certain lines exhibited a relatively higher proportion of polyploid cells (AL-T-BC: 16–27%; G-BC: 11–18%; G-L: 10–14%; PAP-M: 20–26%; PG-M: 20–22%; SE-L: 26–38%) than others in which polyploid cells ranged between 2 and 9%. Over 90% of the polyploid metaphases observed had a normal tetraploid (2 × 46) mode. No difference was found in chromosome preparations made from cultures grown in PRC-1, McCoy's 5a or MEM medium. This observation as well as previous reports [20, 22, 23, 34, 35], signify the tendency to conserve a diploid mode irrespective of the medium being used as a property of the human fibroblast. Slight deviations from this property, however, may be attributed to the tissue origination [18, 23, 26, 34], age of the donor [23, 26, 34], and the time in culture [23, 25, 26].

The maintenance of fibroblastic cells in culture for extended durations may be considered as a different phenomenon from the enhancement of multiplication or clonal growth, since it may involve an influence on the mechanism of the biologically predetermined finite number of generations under in vitro conditions, except the absolute requirements for growth. The chronic effect of trypsin on the cell surface should also be examined as an important parameter of the in vitro conditions. The cell requirements for growth, according to the present and previous studies [2, 35], may involve variations in relative constituent concentrations in the medium. Further modifications of a medium may, therefore, be based on the aspect that an important role is played by the quantitative composition.

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References

1 Paul, J.: Cell and tissue culture (Churchill Livingston, Edinburgh 1975).
2 Litwin, J.: Human diploid cell response to variations in relative amino acid concentrations in Eagle's medium. Expl Cell Res. *72:* 566–568 (1972).
3 Higuchi, K.: Cultivation of animal cells in chemically defined media. Adv. appl. Microbiol. *16:* 111–136 (1973).
4 Ham, R.G.; McKeenan, W.L.: Media and growth requirements. Meth. Enzym. *58:* 44–93 (1979).
5 Ham, R.G.: Survival and growth requirements of nontransformed cells. Hdbook exp. Pharmacol. *57:* 13–88 (1981).
6 Ham, R.G.: Clonal growth of mammalian cells in a chemically defined medium. Proc. natn. Acad. Sci. USA *53:* 288–293 (1965).
7 Shipley, G.D.; Ham, R.G.: Improved medium and culture conditions for clonal growth with minimal serum protein and for enhanced serum-free survival of Swiss 3T3 cells. In Vitro *17:* 656–670 (1981).
8 Delinassios, J.G.: PhD thesis University of Manchester (1974).
9 Delinassios, J.G.; Kottaridis, S.D.; Garas, J.: Uncontrolled growth of tumor stromal fibroblasts in vitro. Expl Cell Biol. *51:* 201–209 (1983).
10 McCoy, T.A.; Maxwell, M.; Kruse, P.F.: Amino acid requirements of the Novicoff hepatoma in vitro. Proc. Soc. exp. Biol. Med. *100:* 115–118 (1959).

11 Eagle, H.: Amino acid metabolism in mammalian cell cultures. Science *130:* 432–434 (1959).
12 Leibovitz, A.: The growth and maintenance of tissue cell cultures in free exchange with the atmosphere. Am. J. Hyg. *78:* 173–180 (1963).
13 Studzinsky, G.P.; Gierthy, J.F.; Cholon, J.J.: An autoradiographic screening test for mycoplasmal contamination of mammalian cell cultures. In Vitro *8:* 466–473 (1973).
14 Morgan, J.F.; Morton, H.J.; Parker, R.C.: Nutrition of animal cells in tissue culture. I. Initial studies on a synthetic medium. Proc. Soc. exp. Biol. Med. *73:* 1–8 (1950).
15 Dulbecco, R.; Freeman, G.: Plaque production by the polyoma virus. Virology *8:* 369–406 (1959).
16 Taylor, W.G.; Dworkin, R.A.; Pumper, R.W.; Evans, V.J.: Biological efficacy of several commercially available peptones for mammalian cells in culture. Expl Cell Res. *74:* 275–279 (1972).
17 Delinassios, J.G.; Voloudakis-Baltatzis, I.; Kottaridis, S.D.; Garas, J.: Nonrandom association of acrocentric chromosomes in human epithelial cells. Experientia *37:* 476–477 (1981).
18 Delinassios, J.G.; Kottaridis, S.D.: Interactions between human lymphoblastoid cells and human fibroblast feeder layers in vitro. Expl Cell Biol. *49:* 177–184 (1981).
19 Delinassios, J.G.; Pandis, N.B.; Margaronis, M.; Kottaridis, S.D.: Double minutes in fibroblast-like cells isolated from human tumors. Experientia *37:* 569–570 (1981).
20 Tjio, J.H.; Puck, T.T.: Genetics of somatic mammalian cells. II. Chromosome constitution of cells in tissue culture. J. exp. Med. *108:* 259–268 (1958).
21 Hayflick, L.; Moorhead, P.S.: The serial cultivation of human diploid cell strains. Expl Cell Res. *25:* 585–621 (1961).
22 Hayflick, L.: The limited in vitro lifespan of human diploid cell strains. Expl Cell Res. *37:* 614–636 (1965).
23 Martin, G.M.; Sprague, C.A.; Epstein, C.J.: Replicative lifespan of cultivated human cells. Effects of donor's age, tissue and genotype. Lab. Invest. *23:* 86–92 (1970).
24 Smith, J.R.; Hayflick, L.: Variation in the lifespan of clones derived from human diploid cell strains. J. Cell Biol. *62:* 48–53 (1974).
25 Matsumura, T.: Multiplication and polyploidization of aging human cells in culture: in Oota, Makinodan, Iriki, Baker, Aging phenomena, pp. 81–109 (Plenum Press, New York 1980).
26 Gabbiani, G.; Rungger-Brändle, E.: The fibroblast; in Glynn, Tissue repair and regeneration, pp. 1–50 (Elsevier/North-Holland, Amsterdam 1981).
27 Pontén, J.: Spontaneous and virus-induced transformation in cell culture. Virol. Monogr. *8:* 253–280 (1971).
28 Pontén, J.: The relationship between in vitro transformation and tumor formation in vivo. Biochim. biophys. Acta *458:* 397–422 (1976).
29 Harper, R.A.; Grove, G.: Human skin fibroblasts derived from papillary and reticular dermis: differences in growth potential in vitro. Science *204:* 526–527 (1979).
30 Azzarone, B.; Macieira-Coelho, A.: Heterogeneity of the kinetics of proliferation within human skin fibroblastic cell populations. J. Cell Sci. *57:* 177–187 (1982).
31 Azzarone, B.; Pedullà, D.; Romanzi, C.A.: Spontaneous transformation of human skin fibroblasts derived from neoplastic patients. Nature, Lond. *262:* 74–75 (1976).
32 Pfeffer, L.; Lipkin, M.; Stutman, O.; Kopelovitch, L.: Growth abnormalities of cultured human skin fibroblasts derived from individuals with hereditary adenomatosis of the colon and rectum. J. Cell Physiol. *89:* 29–38 (1976).
33 Diatloff-Zito, C.; Macieira-Coelho, A.: Effect of growth arrest on the doubling potential of human fibroblasts in vitro: a possible influence of the donor. In Vitro *18:* 606–610 (1982).
34 Hayflick, L.: The longevity of cultured human cells. J. Am. Geriat. Soc. *22:* 1–12 (1974).
35 Waymouth, C.: Rapid proliferation of sublines of NCTC clone 929 (strain L) mouse cells in a simple chemically defined medium (MB 752/1). J. natn. Cancer Inst. *22:* 1003–1017 (1959).

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