Hypothesis

Oxidative stress in cell culture: an under-appreciated problem?

Barry Halliwell*

Department of Biochemistry, Faculty of Medicine, MD 7 #03-07, 8 Medical Drive, Singapore 117597, Singapore

Received 18 January 2003; revised 20 February 2003; accepted 24 February 2003

First published online 11 March 2003

Edited by Robert Barouki

Abstract Cell culture studies have given much valuable information about mechanisms of metabolism and signal transduction and of regulation of gene expression, proliferation, senescence, and death. However, cells in culture may behave differently from cells in vivo in many ways. One of these is that cell culture imposes a state of oxidative stress on cells. I argue that cells that survive and grow in culture might use ROS-dependent signal transduction pathways that rarely or never operate in vivo. A further problem is that cell culture media can catalyse the oxidation of compounds added to them, resulting in apparent cellular effects that are in fact due to oxidation products such as ROS. Such artefacts may have affected many studies on the effects of ascorbate, thiols, flavonoids and other polyphenolic compounds on cells in culture.

Key words: Cell culture; Antioxidant; Free radical; Oxidative stress; Signal transduction

1. Introduction

Cell culture is almost-universally used in laboratories worldwide to examine metabolic pathways and to elucidate the mechanisms involved in signal transduction, regulation of gene expression, cell proliferation and cell death. It has provided a huge amount of valuable information, including helping to elucidate metabolic pathways and the roles of MAP kinases, NFκB, AP-1, nitric oxide and caspases in vivo. However, cells in culture may be different from those in vivo in many ways. To take my own field of interest [1] as an example, oxygen free radicals [including superoxide ($O_2^{\bullet -}$) and hydroxyl ($OH^{\bullet}$) radicals] and other reactive oxygen species (ROS) such as hydrogen peroxide (H_2O_2) have been shown to mediate the growth-promoting, metabolic, or cytostatic effects of a wide range of growth factors and cytokines on cultured cells, ranging from TGFβ to angiotensin II, VEGF, and insulin [2–12]. Yet here there is very limited evidence that these effects mediated by ROS are important in vivo [13–15]. It is well-known that many cellular events are regulated by changes in redox status, often involving the glutathione and thioredoxin systems [5,10,15,16]. ROS can alter the redox status of the cell and thus 'send a signal', but this does not mean that ROS are necessarily the mechanism by which such signals are sent in vivo [13–15]. A huge range of agents, from cytotoxic drugs to diet-derived antioxidants show remarkable effects when added to cells in culture, yet many are a disappointment when tried in vivo. Metabolism and bioavailability are obvious explanations for this discrepancy. I suggest here however that cell culture imposes an inappropriate oxidative stress that may, under certain circumstances, lead to misleading conclusions.

Many cell culture studies are done with malignant cell lines, because such cells are robust and grow and divide easily in culture. This makes sense when looking for chemotherapeutic agents, but less sense when trying to elucidate pathways relevant to normal cells. An alternative is primary culture, where cells are harvested from a tissue and plated. Some of them will survive, but many die because of the stress of the isolation procedure and the 'foreign environment' of the culture conditions. Values of 1–10% have been quoted for the number of originally harvested cells that survive [17]. The words 'culture shock' have been used to describe this phenomenon [17,18]. To take examples, isolated of rat hepatocytes causes activation of nitric oxide synthase within them, generating toxic levels of NO that alter cell metabolism [19]. Cell trypsinisation processes can cause a decrease in levels of cellular reduced glutathione, GSH [20,21]. The cells which do survive 'culture shock' appear to be those that have adapted rapidly, with multiple changes in gene expression, metabolic activity and the levels of enzymes [21–23]. Some enzyme levels are upregulated, other swiftly downregulated [23]. Thus only a fraction, and probably an unrepresentative fraction, of the initially plated cells survives. As an example, p53 is not expressed in 14-day mouse embryos but in culture of fibroblasts from them, the cells that survived were those that had begun to express it [24].

2. 'Culture shock' involves oxidative stress

'Culture shock' affects cells in many ways, but one of them is to impose oxidative stress. The term 'oxidative stress' refers to a serious imbalance between the levels of ROS in a cell and its antioxidant defences in favour of the former [1,25]. Cell culture causes oxidative stress for two reasons: (a) it leads to more ROS generation, and (b) it can impair cellular antioxidant defences.

2.1. More ROS generation

Most cells in vivo in animals are exposed to low O_2 concentrations, in the range of 1–10 mm Hg, although there are

*Fax: (65) 6779 1453.
E-mail address: bchbh@nus.edu.sg (B. Halliwell).

Abbreviations: ROS, reactive oxygen species

0014-5793/03/$22.00
doi:10.1016/S0014-5793(03)00235-7

exceptions (including skin epidermis, cornea and respiratory tract lining cells) [1,26]. Cell culture is commonly performed under 95% air/5% CO_2, approximately 150 mm Hg O_2 tension. The rates of production of ROS by cellular enzyme systems and by 'leakage' of electrons from electron transport chains are O_2-limited at normal cellular levels and will thus increase if O_2 level is raised [1,26–28]. Therefore more ROS will be produced in cells in culture. Cellular oxidative stress can cause senescence, cell death, or adaptation [1]. Cells that fail to adapt may not divide or may die [1,13,29–34] and only the cells that adapt, probably a small proportion of the total, will survive. Even then, the culture stress may affect their properties. An example of this is revealed by attempts to determine the 'Hayflick limit', the number of times that cells can divide in culture before they undergo senescence [17]. For human fibroblasts a value of 40–50 doublings was originally suggested. Recent work by Shay et al. [17] has shown that if cells are grown at low O_2, many more doublings are possible. The original Hayflick limits were artefactually low, due in part to culture-related oxidative stress causing accelerated telomere shortening [17,35]. Others of the many aspects in which cell culture does not replicate conditions in vivo also affect the apparent Hayflick limit [36].

Cellular adaptation to the oxidative stress of 'culture shock' in the minority of cells that survive could, in principle, involve increases in antioxidant defences, downregulating levels of ROS-generating enzymes, adapting electron transport chains to become less leaky and so produce lower amounts of $O_2^{\bullet-}$, or altering cellular targets of oxidative damage to become more resistant to damage by ROS [1,34,37]. Adaptation can be associated with O_2-dependent increased mutation frequency or a hypermutable state [38–40]. A further step might then occur. It is possible that a cell that has adapted to oxidative stress can further adapt to *use* ROS. For example, some malignant cells in culture appear to use ROS to promote proliferation and suppress apoptosis [41,42]. Non-malignant cells may evolve comparable strategies; because more ROS are produced under the pro-oxidant environment of cell culture, cells may activate ROS-dependent signal transduction pathways that rarely, if ever, operate in vivo in healthy tissues. It is thus possible that some of the ROS-dependent pathways that various cells use to respond to cytokines and other molecules in culture [2–11] are not physiological. More work in whole animals is required [13]. For example, overexpression of antioxidant defence enzymes in transgenic animals would be expected to lower ROS and interfere with ROS-dependent signal transduction pathways, but few studies of this type have been reported.

2.2. Fewer antioxidants

Adaptation of cells by upregulation of antioxidant defence systems is also hampered in the 'culture shock' regime. Cell culture media are frequently deficient in antioxidants, especially tocopherols and ascorbate. Vitamin E is rarely added because it is insoluble in water and vitamin C because it is unstable (section 3 below). It follows that cells are deprived of important antioxidants, a situation which can lead to over-interpretations of the beneficial effects of added antioxidants [43–45]. Antioxidants may appear to have beneficial effects when added to cultured cells, but this is because a deficiency is being corrected rather than being a real beneficial effect of 'extra antioxidants'. Indeed, this may help to explain why trials of antioxidant supplementation in humans have given only limited evidence of benefit, although of course many other factors are important in explaining the results of such trials (reviewed in [29]). Culture media can be deficient in selenium [43,46], to an extent which may decrease (or at least prevent oxidative stress-triggered rises in) the activities of anti-

Table 1
Examples of artifacts probably caused by oxidation of compounds added to cell culture media

Observation	Comment	Reference
Induction of apoptosis by ascorbate in HL-60 cells	Entirely due to generation of H_2O_2 by ascorbate oxidation in cell culture media	[52]
Induction of apoptosis by green tea in PC12 cells	Entirely due to generation of H_2O_2 by oxidation of tea components in cell culture media	Halliwell and Long submitted
Induction of cell death by L-DOPA and dopamine in PCl2 and M14 cells	Entirely due to H_2O_2, quinones, and semiquinones generated by oxidation of L-DOPA and dopamine in the culture medium	[70]
Toxicity of apple phenolics to cancer cells	Entirely due to oxidation to produce H_2O_2 in the culture medium	[62]
Cell death induced by gallic acid	Entirely due to oxidation of gallic acid to produce H_2O_2 in culture medium	[60]
Addition of grape seed extract to CaCo-2 cell culture medium generates H_2O_2 due to oxidation of phenolics in the medium	–	[63]
Effects of polyphenols on c-jun phosphorylation in bronchial epithelial cell lines	Shown to involve H_2O_2. Although H_2O_2 was not specifically identified as coming from the culture medium, this seems likely	[64]
Epigallocatechin gallate induces apoptosis in human oral cell lines	Due to production of H_2O_2 in the culture medium	[65]
Toxicity of myricetin to Chinese hamster lung fibroblast V79 cells	Due to H_2O_2 production. Although H_2O_2 was not specifically identified as coming from the culture medium, this seems likely	[66]
Cell culture media found to generate ROS as detected by spin traps and fluorescent dyes	–	[67]
Ascorbate observed to inhibit cell proliferation and fibronectin synthesis in human skin fibroblasts	Inhibition by catalase, suggests may be due to H_2O_2 generation in the culture medium	[52,68]
Inhibition of β-catenin/Tcf activity by tea and epigallocatechin-3-gallate	H_2O_2 generated in the culture medium, but minor contributor to the effects observed	[69]

oxidant systems that are selenium-dependent. These include thioredoxin reductase [16] and the glutathione peroxidase family [46].

3. Cell culture media can be pro-oxidant

Cells require transition metal ions, especially iron and copper, in order to grow. Unless specially purified, all laboratory solutions and cell culture media are contaminated with such ions [1,47–49]. In some media, inorganic metal salts are added. Thus Dulbecco's modified Eagle's medium contains added iron(III) nitrate, $Fe(NO_3)_3$. In other media, iron is supplied in transferrin-bound form, and calf serum will contain some transferrin. Whereas transferrin-bound iron will not normally catalyse free radical reactions [50], 'free' iron ions are powerfully pro-oxidant, as are copper and many other transition metal ions [1,51].

Ascorbate, flavonoids, many other polyphenolic compounds, and thiols are unstable in commonly used cell culture media, undergoing rapid oxidation to generate H_2O_2 and other ROS [52–55]. We have proposed that many of the apparent effects of these molecules on cells in culture are artefacts due to oxidation in the medium. Examples to illustrate this are accumulating rapidly (Table 1). Indeed, the problem may be more widespread. Many substances, including agents used as allegedly specific inhibitors of signal transduction pathways and metabolic pathways, are polyphenolic compounds, and thus likely to be highly oxidisable in cell culture media and prone to generate artefacts if added at high levels (Table 1). On the basis of their structure, examples that may be worthy of investigation include isoproterenol, baicalein, rottlerin, radicicol and the tyrphostin family. Yet another mechanism of pro-oxidant effects is photochemical. If light intensity is sufficiently high, riboflavin in culture media can cause photochemical ROS formation [56,57].

A mainstay of most culture media is foetal calf serum. Serum or plasma contain some antioxidant enzymes, including low levels of catalase, superoxide dismutase, and glutathione peroxidase [58]. Heat treatment, freezing or prolonged storage of serum will inactivate catalase [1]. Breakdown of ceruloplasmin in stored serum proceeds rapidly and releases copper ions [51]. Indeed, leakage of catalase from cells in culture can raise medium levels in some cases, and a human T-cell line was able to grow in culture at high, but not low cell densities for this reason [59]. A 'protective factor' isolated from medium previously used to grow hepatocytes was able to protect other cells against the cytotoxicity of gallic acid; the factor was identified as catalase (Table 1) [60]. Extracellular superoxide dismutase may also be secreted into medium by some cell types [61]. Catalase in the medium can protect against H_2O_2 generated both intracellularly and extracellularly, since H_2O_2 readily crosses cell membranes and so external catalase can 'drain' H_2O_2 out of the cell [1]. The amount and previous treatment of any foetal calf serum present will thus be another variable to consider in cell culture.

4. Conclusion

It is widely realised among experts that cells that survive and grow in culture are not always representative of cells in vivo, in terms of metabolism, gene expression, and enzyme levels and that there is a need for caution in extrapolating data obtained in cell culture to the in vivo situation. Less widely realised is the extent of the oxidative stress that can be caused by the cell culture process. Thus ROS-dependent signal transduction pathways identified in cultured cells need to be validated in knockout or transgenic animals. Similarly, in studies of cellular effects of autoxidisable biomolecules or extracts containing them, it must be realized that ROS production can occur by chemical reactions in the culture media. The stability of such compounds in the culture medium, and their propensity to produce ROS and other oxidation products, must be checked before beginning cell studies. Depending on the cell type and the amount of ROS produced, these species might exert toxic effects. This explains many (Table 1) but not all [69] of the previously reported cellular effects of thiols, ascorbate, and phenols. The resistance of cells to damage by ROS varies widely, depending on the extent to which the cell has adapted to the oxidative stress of the cell culture milieu. Low levels of H_2O_2 can have the paradoxical effect of accelerating proliferation in some cell types [5,10]. Similarly, beware of interpreting what appears to be cellular senescence in cultured cells; rarely are culture conditions adequate to permit cells to reach true replicative senescence [17,36].

Acknowledgements: I am grateful to the National Medical Research Council, the Biomedical Research Council and the Academic Research Fund of the National University of Singapore for grant support.

References

[1] Halliwell, B. and Gutteridge, J.M.C. (1999) Free Radicals in Biology and Medicine, edn. 3, Oxford University Press, Oxford.
[2] Thannickal, V.J. and Fanburg, B.L. (2000) Am. J. Physiol. 279, L1005–L1028.
[3] Woo, C.H., Eom, Y.W., Yoo, M.H., You, H.J., Han, H.J., Song, W.K., Woo, Y.J., Chun, J.S. and Kim, J.H. (2000) J. Biol. Chem. 275, 32357–32362.
[4] Gopalakrishna, R. and Jaken, S. (2000) Free Radic. Biol. Med. 28, 1349–1361.
[5] Droge, W. (2001) Physiol. Rev. 82, 47–95.
[6] Mahadev, K., Wu, X., Zilbering, A., Zhu, L., Laurence, T.R. and Goldstein, B.J. (2001) J. Biol. Chem. 276, 48662–48669.
[7] Touyz, R. and Schiffrin, E.L. (1999) Hypertension 34, 976–982.
[8] Brar, S.S., Kennedy, T.P., Whorton, A.R., Murphy, T.A., Chitano, P. and Hoidal, J.R. (1999) J. Biol. Chem. 274, 20017–20026.
[9] Griendling, K.K. and Harrison, D.G. (1999) Circ. Res. 85, 562–563.
[10] Meier, B. (2001) Protoplasma 217, 101–116.
[11] Steinbeck, M.J., Kim, J.K., Trudeau, M.J., Hauschka, P.V. and Karnovsky, M.J. (1998) J. Cell Physiol. 176, 574–587.
[12] Colavitti, R., Pani, G., Bedogni, B., Anzevino, R., Borrello, S., Waltenberger, J. and Goleotti, T. (2002) J. Biol. Chem. 277, 3101–3108.
[13] Halliwell, B. (1996) Biochem. Soc. Trans. 24, 1023–1027.
[14] Bowie, A. and O'Neill, L.A.J. (2000) Biochem. Pharmacol. 59, 13–23.
[15] Bajt, M.L., Ho, Y.S., Vonderfecht, S.L. and Jaeschke, H. (2002) Antioxid. Redox Signal. 4, 733–740.
[16] Tanaka, K., Nakamura, H., Nishiyama, A., Hosoi, F., Masutani, H., Wada, H. and Yodoi, J. (2001) Free Radic. Res. 33, 851–855.
[17] Wright, W.E. and Shay, J.W. (2002) Nat. Biotechnol. 20, 682–688.
[18] Sherr, C.J. and De Pinho, R.A. (2000) Cell 102, 407–410.
[19] Lopez-Garcia, M.P. and Sanz-Gonzalez, S.M. (2000) FEBS Lett. 466, 187–191.
[20] Reiners Jr., J.J., Mathieu, P., Okafor, C., Putt, D.A. and Lash, L.H. (2000) Toxicol. Lett. 115, 153–163.
[21] Bishop, C.T., Mirza, Z., Crapo, J.D. and Freeman, B.A. (1985) Cell Dev. Biol. 21, 229–236.

[22] Tu, B., Wallins, A., Moldeus, P. and Cotgreave, I.A. (1994) Pharmacol. Toxicol. 75, 82–90.
[23] Baker, T.K., Carfagna, M.A., Gao, H., Dow, E.R., Li, Q., Searfiss, G.H. and Ryan, T.P. (2001) Chem. Res. Toxicol. 14, 1218–1231.
[24] Mendrysa, S.M. and Perry, M.E. (2000) Mol. Cell Biol. 20, 2023–2030.
[25] Sies, H. (1999) Oxidative Stress II, Oxidants and Antioxidants, Academic Press, New York.
[26] De Groot, H. and Littauer, A. (1989) Free Radic. Biol. Med. 173, 541–551.
[27] Yusa, T., Crapo, J.D. and Freeman, B.A. (1984) Biochim. Biophys. Acta 798, 167–174.
[28] Turrens, J.F., Freeman, B.A., Levitt, J.G. and Crapo, J.D. (1982) Arch. Biochem. Biophys. 217, 401–410.
[29] Halliwell, B. (2000) Lancet 355, 1179–1180.
[30] Chen, Q.M., Liu, J. and Merrett, J.B. (2000) Biochem. J. 347, 543–551.
[31] Burdon, R.H. (1995) Free Radic. Biol. Med. 18, 775–794.
[32] Davies, K.J.A. (1999) IUBMB Life 48, 41–47.
[33] Aerts, C., Wallaert, B. and Voisin, C. (1992) Exp. Lung Res. 18, 845–861.
[34] Joenje, H., Gille, J.J., Oostra, A.B. and Van der Valk, P. (1985) Lab. Invest. 52, 420–428.
[35] Von Zglinicki, T. (2002) Trends Biol. Sci. 27, 339–344.
[36] Ramirez, R.D., Morales, C.P., Herbert, B.S., Rohde, J., Passons, C., Shay, J.W. and Wright, W.E. (2001) Genes Dev. 15, 398–403.
[37] Barja, G., Cadenas, S., Rojas, C., Perez-Campo, R. and Lopez-Torres, M. (1994) Free Radic. Res. 21, 317–327.
[38] Kamijo, T., Zindy, F., Roussel, M.F., Quelle, D.E., Downing, J.R., Ashmun, R.A., Grosveld, G. and Sherr, C.J. (1997) Cell 91, 649–659.
[39] Gille, G.J. and Joenje, H. (1989) Mutat. Res. 219, 225–230.
[40] Bruyninckx, W.J., Mason, H.S. and Morse, S.A. (1978) Nature 274, 606–607.
[41] Clement, M.V. and Pervaiz, S. (1999) Free Radic. Res. 30, 247–252.
[42] Liu, R., Li, B. and Qiu, M. (2001) Oncogene 20, 1486–1496.
[43] Leist, M., Raab, B., Maurer, S., Rosick, U. and Brigelius-Flohe, R. (1996) Free Radic. Biol. Med. 21, 297–306.
[44] Nowak, G. and Schnellmann, R.G. (1996) Am. J. Physiol. 271, C2072–C2080.
[45] Smith, A.R., Visioli, F. and Hagen, T.M. (2002) FASEB J. 16, 1102–1104.
[46] Baker Jr., R.D., Baker, S.S. and Rao, R. (1998) J. Pediat. Gastroenterol. Nutr. 27, 387–392.
[47] Biaglow, J.E., Issels, R.W., Gerweck, L.E., Varnes, M.E., Jacobson, B., Mitchell, J.B. and Russo, A. (1984) Radiat. Res. 100, 298–312.
[48] Messer, H.H., Murray, E.J. and Goebel, N.K. (1982) J. Nutr. 112, 652–657.
[49] Evans, P.J., Tredger, J.M., Dunne, J.B. and Halliwell, B. (1996) Transplantation 62, 1046–1049.
[50] Aruoma, O.I. and Halliwell, B. (1987) Biochem. J. 241, 273–278.
[51] Gutteridge, J.M.C., Winyard, P.G., Blake, D.R., Lunec, J., Brailsford, S. and Halliwell, B. (1985) Biochem. J. 230, 517–523.
[52] Clement, M.V., Jeyakumar, R., Long, L.H. and Halliwell, B. (2001) Antiox. Redox Signal. 3, 157–164.
[53] Halliwell, B., Clement, M.V., Ramalingam, J. and Long, L.H. (2000) IUBMB Life 50, 251–257.
[54] Long, L.H., Clement, M.V. and Halliwell, B. (2000) Biochem. Biophys. Res. Commun. 273, 50–53.
[55] Long, L.H. and Halliwell, B. (2001) Biochem. Biophys. Res. Commun. 286, 991–994.
[56] Zieger, M.A., Glofcheski, D.J., Lepcock, J.R. and Kruuv, J. (1991) Cryobiology 28, 8–17.
[57] Wang, R.J. and Nixon, B.R. (1978) In Vitro 14, 715–722.
[58] Halliwell, B. and Gutteridge, J.M.C. (1990) Arch. Biochem. Biophys. 280, 1–8.
[59] Sandstrom, P.A. and Buttke, T.M. (1993) Proc. Natl. Acad. Sci. USA 90, 4708–4712.
[60] Isuzugawa, K., Inone, M. and Ogihara, Y. (2001) Biol. Pharm. Bull. 24, 1022–1026.
[61] Yamada, H., Adachi, T., Fukatsu, A., Misao, S., Yamada, Y., Aoki, T., Miura, N., Sakuma, M., Nishikawa, K., Futenma, A. and Kakumu, S. (2002) FEBS Lett. 519, 77–81.
[62] Lapidot, T., Walker, M.D. and Kanner, J. (2002) J. Agr. Food Chem. 50, 3156–3160.
[63] Roques, S.C., Landrault, N., Teissedre, P.L., Laurent, C., Besancon, P., Rovanet, J.M. and Caporiccio, B. (2002) Free Radic. Res. 36, 593–599.
[64] Yang, G.Y., Liao, J., Li, C., Chung, J., Yurkow, E.J., Ho, C.T. and Yang, C.S. (2000) Carcinogenesis 21, 2035–2039.
[65] Sakagami, H., Arakawa, H., Maeda, M., Satoh, K., Kadofuku, T., Fukuchi, K. and Gomi, K. (2001) Anticancer Res. 21, 2633–2642.
[66] Kajiya, K., Ichiba, M., Kuwabara, M., Kumazawa, S. and Nakayama, T. (2001) Biosci. Biotechnol. Biochem. 65, 1227–1229.
[67] Bartosz, G. (2000) Acta Biochim. Pol. 47, 1197–1198.
[68] Peterszegi, G., Dagonet, F.B., Labat-Robert, J. and Robert, L. (2002) Eur. J. Clin. Invest. 32, 372–380.
[69] Dashwood, W.M., Orner, G.A. and Dashwood, R.H. (2002) Biochem. Biophys. Res. Commun. 296, 584–588.
[70] Clement, M.V., Long, L.H., Ramalingam, J. and Halliwell, B. (2002) J. Neurochem. 81, 414–421.